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(54) **Recombinant fowlpox virus with intact FPV-tk-gene.**

(57) An improved method is described to prepare recombinant fowlpox virus for the expression of proteins or for use as a vaccine. The new method uses for the insertion of foreign DNA an intergenic region which is located between the FPV thymidine kinase (tk) gene and the 3'-open reading frame. Said intergenic region is enlarged to comprise one or more unique restriction sites, thereby allowing insertion of foreign DNA in such a way that the FPV tk-gene remains intact and codes for the entire thymidine kinase.

New strong poxvirus promoters are presented and new FPV host virus strains carrying a vaccinia virus thymidine kinase gene and an E. coli lacZ gene as a novel non-essential site. The novel fowlpox virus host strains allow the use of any insertion plasmid carrying vaccinia virus tk-gene flanking regions.

The invention concerns recombinant fowlpox virus (FPV), specific vectors, new strong promoters, novel FPV host strains as well as a process for the recombinant production of proteins.

Fowlpox virus, the archetypal member of the avian pox viruses, possesses the typical pox virus structure. The viral genome has been estimated to be  $200-240 \times 10^6$  daltons.

Pox of birds, though prevalent world-wide, is not considered a public health problem because the host-range of the avian pox viruses is limited to birds and excludes mammals. After infection of a host, viral DNA replication starts, after an early protein synthesis, between 60 and 96 hours post-infection and is followed by the synthesis of late proteins. The assembly of infectious virions occurs between 72 and 96 hours.

Growth of FPV in tissue culture cells has been achieved on chicken embryo fibroblast (CEF) cells, chicken embryo dermal (CED) cells as well as duck embryo fibroblast (DEF) cells. In tissue culture, the viral cycle is similar and appears to be quicker than in birds. In the CED cells DNA replication commences between 12 and 16 hours, and infectious virus particles first appear after 16 hours and continue to increase in number until 48 hours post-infection.

For vaccinia virus (VV), the archetypal member of the orthopox virus, Panicali & Paoletti (Proc. Natl. Acad. Sci., 79, 4927-4931 (1982)) as well as Mackett et al (Proc. Natl. Acad. Sci., 79, 7415-7419 (1982)) have developed a technique known as in vivo recombination which allows the insertion of foreign DNA into the vaccinia virus genome by site-specific recombination. This technique has led to the use of vaccinia virus as a eukaryotic expression vector for creating live recombinant vaccines. The construction of recombinant pox viruses is usually done by insertion of foreign genes into regions of the viral genome that are non-essential for growth in cell culture. For recombinant vaccinia viruses the thymidine kinase (tk) gene is such a non-essential site (NES) that, in addition, allows selection of tk-negative recombinant viruses.

For the construction of recombinant FPV the same principles are applied as described for recombinant vaccinia virus. Several non-essential sites have been described including the fowlpox virus thymidine kinase gene in the strain FPV-M3 (Boyle & Coupar, PCT/AU87/00323; Boyle & Coupar, Virus Res., 10, 343 (1988)), a region present on a 900 bp PvuII fragment of the wild-type virus strain FP-I (Taylor et al, Vaccine, 6, 497-503, 504-508 (1988)), and the intergenic region between the open reading frames orf 7 and orf 9 (Drillien et al, Virology, 160, 203-209 (1987); Spehner et al, J. Virol., 64, 527-533 (1990)).

Recently, several groups have described the construction of FPV recombinants. Noboru et al disclose in EP-284,416 a number of genomic insertion sites which are non-essential for FPV growth in tissue culture. Paoletti describes in PCT/WO-89/03429 vectors for producing FPV recombinants; they disclose the expression of genes encoding foreign antigens under the control of various vaccinia promoters.

Further, Binns et al disclose in PCT/WO-90/04638 a number of FPV promoters using a transient assay with  $\beta$ -galactosidase. Drillien and Spehner describe in EP-314,569 the construction of FPV recombinants containing a gene which encodes the measles F protein under the control of a vaccinia promoter. The gene was inserted into the FPV genome at a site non-essential for growth in tissue culture.

Cohen and Panicali describe in PCT/WO-90/02191 a recombinant fowlpox virus capable of expressing immunogenic protein of pathogens. This recombinant FPV provides a live vaccine for poultry and other animals.

The present inventors have realized that the presence of an intact thymidine kinase gene is required in the FPV strain HPI.441 to obtain stable recombinants with predictable genomes.

Up to date it has not been clarified scientifically to what an extent the tk-gene is essential for various FPV strains. To overcome this uncertainty, the inventors have searched for further locations to insert foreign DNA, and have found that the intergenic region between the intact tk-gene and the 3' open reading frame is a preferable insertion site. The present invention also provides novel FPV host strains, which have been modified to comprise a vaccinia virus thymidine kinase gene and an E. coli lacZ gene as a novel non-essential site, thereby allowing the use of any insertion plasmid which carries vaccinia virus tk flanking regions. The invention also provides new strong promoters as well as a number of preferred plasmid constructs.

To show the advantages according to the invention, FPV insertion plasmids were constructed which use as the site of insertion of the foreign marker gene either the interrupted viral thymidine kinase gene or the intergenic region between the intact tk-gene and the 3' open reading frame. The analysis of the genomic structures of the recombinants derived from both types of experiments revealed that only in the presence of an intact thymidine kinase gene stable recombinants with predictable genomes were obtained. This result strongly suggests that the FPV tk-gene is essential in its entirety for growth of the virus in cell culture.

Brief description of the Figures:

Fig. 1

5 Construction schemes of the fowlpox virus insertion plasmids pFP-UV2 and pFP-UV2-PT. The abbreviations have the following meaning:  
 FPV-tk = fowlpox virus thymidine kinase gene; VV-tk = vaccinia virus thymidine kinase gene; PII = promoter of the vaccinia virus major late 11kDa polypeptide; P7.5 = promoter of the vaccinia virus 7.5kDa polypeptide; lacZ = E.coli gene encoding  $\beta$ -galactosidase (the arrows indicate the directions of transcrip-  
 10 tion).

Fig. 2A - 2C

15 Southern blot analysis of FPV recombinants derived from the insertion plasmid pFP-UV2-PT. Total DNA was prepared from infected cells, digested with EcoRI, separated on a 1 % agarose gel and transferred onto a nitrocellulose membrane. The membrane was hybridized to a <sup>32</sup>P labeled FPV tk-gene probe (Fig. 2A), a lacZ gene probe (Fig. 2B) and a prothrombin gene probe (Fig. 2C). Lanes 1-5 in all figures represent DNAs from the FPV recombinant f-PTI-blue at different stages of plaque purification (3rd, 5th, 7th, 9th and 11th round, respectively). In lane 6 a different isolate, f-PT2-blue, is shown. Lanes 7, 8 and 9 represent FPV  
 20 wild-type and two independent white plaque isolates (f-PT-white 1 and 2), respectively. As a negative control chicken embryo fibroblast DNA is shown in lane 10. The arrowhead in Fig. 2A points to the FPV wild-type tk-gene band. The values given on the right correspond to standards in kilo basepairs (kb).

Fig. 3A

25 Structure of the wild-type and the mutant fowlpox virus tk-locus. The locations of the FPV tk-gene in the 5.5 kb <sup>\*</sup>FcoRI fragment and in the 2.48 kb BamHI/ClaI fragment are shown. (The single NcoI site in the middle of the coding region of the tk-gene was used to construct the insertion vector pFP-UV2.)

Immediately downstream of the tk-gene, the intergenic region was modified and enlarged by  
 30 oligonucleotide directed mutagenesis leaving the 3'orf and the tk-gene itself intact while introducing a transcription stop signal and several convenient restriction sites.

Fig. 3B

35 Sequence of the FPV wild-type and of the modified intergenic region. The modified intergenic region is present in the recombination plasmid pTKm and its derivatives.

Fig. 4A and 4B

40 Construction of the FPV insertion plasmids pTKm-sPII-gpt, pTKm-VVtka and pTKm-VVtkb. The details of the constructs are described in the Experimental Part. sPII = synthetic vaccinia virus late promoter derived from the promoter of the vaccinia major late 11kDa polypeptide; 3'orf = open reading frame downstream of the fowlpox virus tk-gene; gpt = E. coli gene coding for the enzyme xanthine-guanine-phosphoribosyl transferase. Further abbreviations are the same as described in the legend of Fig. 1. Arrows  
 45 indicate the direction of transcription.

Fig. 5

50 Southern blot analysis of EcoRI-digested DNA of the purified FPV recombinants f-sPII#I and of FPV wild type virus DNA.

A) The blot was hybridized with the FPV tk-gene probe. Lane 1, DNA of the FPV recombinant f-sPII#I; lane 2, DNA of the FPV wild type virus HPI.441; lane 3, lambda DNA digested with Hind III.

B) The blot was hybridized with the gpt-gene probe; lane 1, lambda DNA digested with HindIII; lane 2, DNA of the FPV recombinant f-sPII#I; lane 3, DNA of the FPV wild type virus HPI.441.

55 C) The blot was hybridized with the lacZ gene and phage lambda DNA probes; lane 1, lambda DNA digested with Hind III, lane 2, DNA of the FPV recombinant f-sPII#I; lane 3, DNA of the FPV wild type virus HPI.441. (The values given on the right correspond to standards in kilo basepairs.)

Fig. 6A - 6C

Southern blot analysis of DNA of the FPV recombinants f-TK2a and f-TK2b. The blots were hybridized with the FPV tk-gene probe (Fig. 6A), the VV-tk gene probe (Fig. 6B) and with lacZ gene and the lambda DNA probes (Fig. 6C). Lanes 1, FPV wild type DNA (HPI.441) digested with PstI; lanes 2, f-TK2a DNA digested with PstI; lanes 3, f-TK2b DNA digested with PstI; lanes 4, FPV wild type DNA (HPI.441) digested with ClaI; lanes 5, f-TK2a DNA digested with ClaI; lanes 6, f-TK2b DNA digested with ClaI; lanes 7, FPV wild type DNA (HPI.441) digested with EcoRI; lanes 8, f-TK2a DNA digested with EcoRI; lanes 9, f-TK2b DNA digested with EcoRI; lanes 10, marker DNA; lanes 11, pTKm-VVtk DNA digested with EcoRI; lanes 12, pTKm-VVtkb DNA digested with EcoRI; lanes 13, pTKm-VVtk DNA digested with ClaI; lanes 14, pTKm-VVtkb DNA digested with ClaI; lanes 15, pTKm-VVtk DNA digested with PstI.

Fig. 6D

The restriction enzyme cleavage maps of the fowlpox virus host strain f-TK2a with the enzymes EcoRI, PstI and ClaI are shown. The numbers indicate the predicted size of the fragments in kilo basepairs (kb). FPV-tk = fowlpox virus thymidine kinase gene; VV-tk = vaccinia virus thymidine kinase gene; sPIL = synthetic PIL promoter; lacZ = E. coli lacZ gene; the arrows indicate the direction of transcription.

Fig. 6E

The restriction enzyme cleavage maps of the fowlpox virus host strain f-TK2b with the enzymes EcoRI, PstI and ClaI are shown. For further information and abbreviations see legend of Fig. 6D.

Fig. 7

Schematic presentation of the region around of the FPV thymidine kinase (tk) locus of the wild type virus and of the novel FPV host strains f-TK2a and f-TK2b. The FPV host strains have inserted into the intergenic region between the FPV tk-gene and the 3'-open reading frame (orf) two new inserts, the vaccinia virus tk-gene (VV-tk) and the E. coli lacZ gene (lacZ). The arrows indicate the direction of transcription of the respective genes.

Fig. 8

Construction scheme of the "promoter trap" plasmids pFP-ZI and pFP-Z2I. The plasmids were constructed as outlined in the graph and as described in the Experimental Part. FPV-tk = fowlpox virus thymidine kinase gene; P7.5 = promoter of the vaccinia virus 7.5kDa protein gene; PIL = promoter of the gene of the vaccinia virus 11kDa polypeptide; ssDNA = single strand DNA; arrows indicate the direction of transcription.

Fig. 9

Construction scheme of the vaccinia virus insertion plasmids pTZgpt-P2a and pTZgpt-P2b. Details of the construction are shown in the graph and described in the Experimental Part. Part of the abbreviations are described in the legend of Fig. 1; gpt = gene coding for the E.coli xanthine guanine phosphoribosyltransferase; arrows indicate the direction of transcription.

Fig. 10A

Sequence of the FPV P2 promoter and the first ten codons of the P2 gene. The A residue of the initiation codon (in bold type) was defined as position +1. At position -6 to -2 the vaccinia virus late promoter core sequence is present and at position -19 to -13 there is a vaccinia early RNA stop signal. Sequences that match in a minimum of 11 nucleotides to the 16 bp vaccinia early promoter critical region are underlined. The upstream region extends up to position -174. The downstream region (30 nucleotides coding sequence of the P2 gene) extends from +1 to +30.

Fig. 10B

Sequence of the NsiI-EcoRI fragment containing the FPV P2 promoter, the P2 gene and the downstream region. For underlined parts see legend to Fig. 10A. The upstream region extends up to position -174. The coding sequence of the P2 gene extends from +1 to +399 and codes for 133 amino acids. The calculated molecular mass of the P2 gene is 14 806 Da. The downstream region (415 bp) is A- and T-rich and does not contain open reading frames coding for proteins larger than 4kDa.

Fig. 11

Comparison of the P2 promoter with other poxvirus promoters. The histogram shows the  $\beta$ -galactosidase expression levels induced by different poxvirus promoter-lacZ constructs. Cytoplasmic extracts of CV-1 cells infected with the indicated recombinant viruses were prepared and assayed for enzymatic activity as described in the Experimental Part. The expression levels of the different recombinants were compared with the standard level of vFls $\beta$  (100 %).

Fig. 12

SDS-PAGE analysis of CV-1 cells infected with different vaccinia recombinants. Cells were infected as described in the Experimental Part. Total soluble proteins were prepared and different amounts (5  $\mu$ l and 10  $\mu$ l) analyzed on a 10 % polyacrylamide gel. Lanes 1 and 2: protein induced by vaccinia wild-type virus; lanes 3 and 4: proteins induced by the vaccinia recombinant vFls $\beta$ ; lanes 5 and 6: proteins induced by the virus vP2a; lanes 7 and 8: proteins induced by the VV recombinant vP2b; lanes 9 and 10: proteins induced by the VV recombinant vart. The reference virus vFls $\beta$  (lanes 3 and 4) induces a novel protein in the 117kDa range (lower arrow) that cannot be detected in wild-type virus infected cells (lanes 1 and 2). The  $\beta$ -galactosidase/P2-gene fusion protein obtained with the recombinants vP2a and vP2b (lanes 5 to 8) is about 130 kDa (upper arrow).

Fig. 13A

Construction scheme of the insertion plasmid pFSgpt. The plasmids were constructed as outlined in the figure. For abbreviations see legend of Fig. 1.

Fig. 13B

Sequence of the multiple cloning site of pFSgpt. The translational stop codons are in bold type; the poxvirus early transcription stop signal is underlined.

Fig. 14A

Construction of the insertion plasmids pP2mxgpt, containing mutated P2 promoter (mx) sequences. Oligonucleotides encoding either wild-type or mutant P2 promoter sequences were ligated into pFSgpt. The E.coli lacZ gene was placed downstream of the various promoters, thereby creating the promoter test plasmids pP2mxgpt-lacZ. P2mx.1 and P2mx.2 = synthetic linker sequences encoding P2 promoter. For further abbreviations see legend of Fig. 1.

Fig. 14B

Sequence of the multiple cloning site of the insertion plasmid pP2mxgpt. The translational start and stop codons are in bold type; the poxvirus early transcription stop signal is underlined.

Fig. 15

Structure of wild-type and mutant P2 promoters.

Fig. 16

Comparison of the  $\beta$ -galactosidase activities induced by the P2 promoter mutants in infected CV-1 cells.

- a) late promoter activities
- b) early promoter activities.

Fig. 17A and 17B

A) Construction scheme of the vaccinia virus insertion plasmids pTZgpt-Fls and pTZgpt-P1IM. The plasmids were constructed as described in the Experimental Part. tk = vaccinia virus thymidine kinase gene; P7.5 = promoter of the gene of the vaccinia virus 7.5kDa protein; P1I = promoter of the gene of the vaccinia virus 11kDa polypeptide; P1IM = mutated P1I promoter; fl ori = fl origin of replication; gpt = E.coli gpt gene (coding for the enzyme xanthine guanine phosphoribosyl transferase); MCS = multiple cloning site.

B) Construction scheme of the promoter test vectors pTZgpt-sP1I, pTZgpt-s4b and pTZgpt-sart (pTZgpt-sPx). FPV-tk = thymidine kinase gene of fowlpox virus; P7.5 = promoter of the gene of the vaccinia virus 7.5kDa protein; -sPx designates the respective synthetic linker sequences sP1I, S4b and sart used for construction of promoters; gpt = E.coli gpt gene coding for xanthine guanine phosphoribosyl transferase; arrows indicate the direction of transcription.

Fig. 18

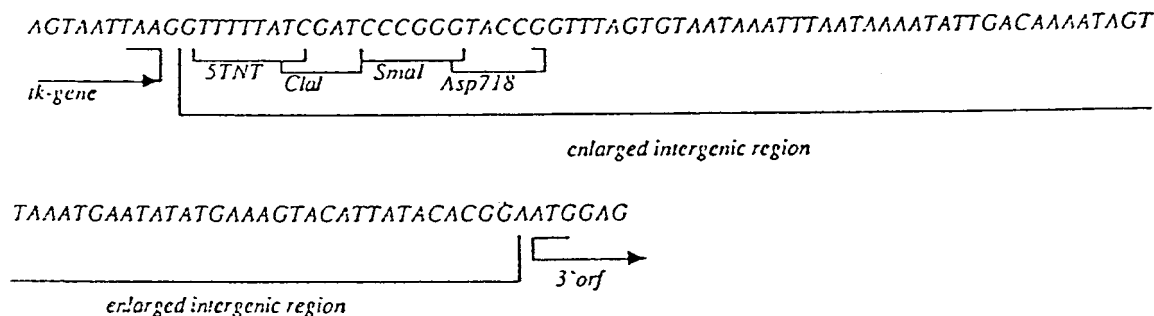
Structure of the promoter regions. The nucleotide sequence of the mutated promoter regions is shown. The vaccinia virus late promoter consensus sequence (thin line), the translation initiation codons (bold lines) and the position of several restriction sites are indicated. P1Iwt, wild-type sequence of the P1I promoter; P1Im, mutated P1I sequence; sP1I, synthetic mutated P1I sequence; s4b, synthetic FPV 4b promoter; sart, synthetic (artificial) late promoter.

Fig. 19

Comparison of  $\beta$ -galactosidase expression levels induced by the different pox virus promoter lacZ gene constructs. The expression levels of the different recombinants were compared with the standard level of vFls $\beta$  (100 %).

The present invention thus concerns a recombinant fowlpox virus (FPV) insertion plasmid, which is characterized in that the intergenic region between the FPV tk-gene and the 3' open reading frame (3' orf) is enlarged to form one or more unique restriction sites so that by insertion of foreign DNA into this intergenic region the FPV tk-gene remains intact and codes for the entire thymidine kinase (TK).

Said enlarged intergenic region may e.g. comprise the following sequence:



This modification of the wild type intergenic region may be obtained by site-specific mutagenesis.

A recombinant FPV capable of expressing foreign protein(s) is produced by integrating into the fowlpox viral genome a DNA sequence encoding foreign protein(s). This foreign DNA sequence is integrated into the FPV genome by an in vivo recombination event between the insertion plasmid carrying the foreign DNA sequence and flanking sequences of the FPV genome. This insertion plasmid comprises at least the foreign DNA sequence linked to a fowlpox or other pox virus promoter located between DNA sequences which are homologous to the above intergenic region and the flanking sequences. Thus, a selectable insertion plasmid

comprises at least:

- (a) a natural or synthetic poxvirus promoter linked to a foreign DNA sequence which is to be expressed;
- (b) a second pox virus promoter linked to a gene encoding a marker or indicator for selection of recombinant FPV;
- (c) DNA sequences of FPV flanking the construct of elements (a) and (b) at both 5' and 3' ends, said flanking DNA sequences being homologous to the sequences upstream and downstream of the enlarged intergenic region.

Above plasmid preferably comprises further a replicon for replication in a prokaryotic host, and a gene encoding a selectable marker or indicator for selection in a transformed prokaryotic host.

The promoters used in the above plasmid as well as in recombinant FPV are pox virus promoters, especially FPV promoters. For efficient expression of a foreign protein it is preferable that the promoter is immediately adjacent to the coding sequence of the foreign DNA sequences.

Most of the VV recombinants constructed so far use cloned VV promoters to drive the foreign gene of interest. In vivo recombination of a transcription unit consisting of a cloned VV promoter and a foreign gene into a non-essential site of the VV genome usually results in the duplication of the promoter elements and may give rise to secondary recombinations, segregation and instability of the recombinant. For the construction of genetically stable pox virus recombinants it is therefore desirable to use either non-homologous or short synthetic viral promoters that control the transcription of the foreign gene.

A preferred FVP promoter is the P2 promoter (Fig. 10B). This promoter contains in its upstream part several critical early regions followed by the late promoter consensus sequence. The functional analysis confirmed that the P2 promoter is active early and late in the viral life cycle.

The strength of the novel FPV promoter was compared with several known strong poxvirus promoters in vaccinia virus recombinants. It was found that the P2 promoter belongs to one of the strongest natural promoters in VV infected cells.

In an attempt to optimize the P2 promoter, a series of mutants was constructed (Fig. 15). In all mutations the P2 gene fusion sequence is removed and the initiation codon of the lacZ gene is located adjacent to the late promoter signal TAAAT. In the mutation mO (TAAATG AAT TCC) the ATG of the lacZ gene is directly fused with the late promoter core sequence, thereby deleting the C residue at position -1 of the wild-type P2 sequence, a mutation that improves the efficiency of a late promoter. This structure is found in many late VV promoters and is thought to be the optimal context of the late promoter consensus and the initiation codon (Davidson, A.J. and Moss B., J. Mol. Biol. 210: 749, 1989).

The mutant m1 (TAAACATG AAT TCC) has the ATG of the lacZ gene directly fused with the ATG of the putative P2 gene.

The mutation m2 was constructed in order to investigate the significance of the early promoter critical regions found upstream of the late promoter region. The mutant promoter m2 has the same structure as m1, except that the early RNA stop signal within the functionally important T-rich region upstream of the late promoter motif was inactivated by a TTG insertion at position -18.

Thus, preferred FPV promoters are the P2 promoter having a DNA sequence as derivable from Fig. 10A and functional equivalents thereof. Experimental data as to the promoter strength are shown in Fig. 16.

The promoter regions are preferably followed by a multiple cloning site (MCS) which allows the insertion of foreign genes.

The P2 gene and the downstream region were characterized by sequence analysis (Fig. 10B). The P2-gene codes for 133 amino acids; the calculated molecular mass is 14 806 Da. The downstream region (415 bp) is A and T rich and does not contain open reading frames coding for proteins larger than 4 kDa, i.e. this region of the genome is probably a non-coding region. The downstream region of the P2 gene is therefore a novel non-essential site that can be used for the insertion of foreign genes into the FPV genome.

Preferred plasmids contain genetic elements which allow selection of recombinant FPV. These elements comprise a gene encoding a selectable marker or indicator together with a poxvirus promoter which controls the expression of said gene in the recombinant virus. The promoter and the marker or indicator gene are located between the flanking FPV sequences so that the same are co-integrated into the FPV genome. Recombinant FPV can then be selected based upon expression of the marker or indicator.

A preferred gene for identification is the E.coli lacZ gene which encodes the enzyme  $\beta$ -galactosidase. Methods for identification based upon expression of this enzyme are discussed in the literature. Selection methods include drug resistance selection, e.g. the selection by the gene encoding xanthine guanine phosphoribosyl transferase, the latter conferring resistance to mycophenolic acid.

The plasmids according to the invention also contain preferably a replicon for replication in a prokaryotic host as well as a gene encoding a selectable indicator or marker which allow selection and amplification in a prokaryotic host such as E.coli. The replicon can be obtained from any conventional

prokaryotic plasmid such as pBR322. The selectable marker can be a gene conferring antibiotic resistance.

Specific plasmids according to the invention may be constructed by replacing the lacZ gene of insertion plasmid pTKm-sPIL-gpt by a foreign gene of interest.

The DNA plasmids containing the DNA sequence to be expressed together with the marker or indicator genes are flanked by appropriate FPV sequences, the latter allowing recombination with FPV and integration of the flanked genes into the FPV genome. This recombination occurs in the cytoplasm of a eukaryotic host cell. Appropriate host cells for recombination require that they are (1) infectable by FPV and (2) transfectable by the DNA vector. Examples of such cells are chicken embryo fibroblast and chicken embryo dermal cells.

For in vivo recombination, the cells are first infected with FPV and then transfected with the insertion plasmid. Viral infection is accomplished by standard techniques for infection of eukaryotic cells with FPV. Subsequently, the cells are transfected with the insertion plasmid by means of any of the conventional transfection techniques.

After infection and subsequent transfection, the cells are incubated under standard conditions, and virus is allowed to replicate; during this time in vivo recombination occurs between the homologous FPV sequences of the insertion vector and FPV so that the foreign DNA sequences are inserted into the FPV genome.

Recombinant FPV is then selected by means of the inserted marker or indicator, e.g. the E.coli lacZ gene which expresses  $\beta$ -galactosidase. Using a chromogenic substrate for this enzyme, e.g. 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside, recombinant viruses are detected as blue plaques.

According to another essential embodiment of the invention, the recombinant FPV comprises as insertion site within the above intergenic region a vaccinia virus tk-gene which can serve as non-essential site (NES) for the insertion of one or more foreign DNA sequences.

As a preferred modification, said recombinant FPV comprises in said enlarged intergenic region a selection marker and/or a reporter gene and the VV tk-gene in any desirable order.

The most preferred modifications consist of recombinant fowlpox viruses which comprise within an enlarged intergenic region the insertion of the vaccinia virus tk-gene and of the lacZ gene. The genomic structures of such two novel host strains is shown in Fig. 7. Either the fowlpox virus or the vaccinia virus tk-gene may be used as non-essential sites to insert foreign genes. The strains f-TK2a and f-TX2b differ only in the orientation of the vaccinia virus tk-gene. This allows the insertion by homologous recombination of foreign genes of interest in two orientations. This may be advantageous to study transcriptional interference phenomena.

Since the above modification of a novel FPV host strain comprises two intact tk-genes, it is possible to use either one for the insertion of foreign DNA. This allows the application of an extended range of plasmids which possess either FPV tk or VV tk flanking sequences.

Thus the invention comprises recombinant FPV which has been obtained by homologous recombination of the above novel FPV host strain and any of the plasmids described here which allow insertion of a foreign DNA into either the FPV tk-gene or the VV tk-gene.

As described above, a recombinant FPV capable of expressing foreign protein(s) is produced by integrating into the FPV genome a DNA sequence encoding said foreign protein(s). This is done by in vivo recombination by means of an insertion vector as described above. Specific vectors according to the invention may be constructed by means of insertion plasmids pTZgpt-FIs or pTZgpt-PIIM as shown in Fig. 17A, and pP2mxgpt as shown in Fig. 14A.

The construct pTZgpt-FIs (Fig. 17A) presents a plasmid that is advantageous in comparison to the previously used plasmid pTXgpt-FIs (upper part of Fig. 17A) in that the fl origin of replication (fl ori) was introduced by substituting the pTZ portion in place of the pUC portion (PvuII fragments). Insertion of the fl ori allows the production of a single-stranded DNA as required for sequencing and in vitro mutagenesis. In this way, time-consuming recloning experiments in M13 vectors are superfluous.

In the plasmid pTZgpt-PIIM (Fig. 17A), the PII "late promoter consensus region" TAAATGAATTC is mutated and converted to the following sequence: TAAATAAAGAATTC. This construct has the advantage that the genes can be expressed under the control of their own translation-initiation codons (ATG).

The plasmid pTZgpt-dP (Fig. 17A) comprises besides the flanking VV tk sequences and the gpt gene under the control of promoter P7.5 a single HpaI site. This site serves conveniently for the insertion of various promoter-foreign-gene cassettes.

The insertion plasmids pP2m0gpt, pP2m1gpt, pP2m2gpt (pP2mxgpt; Fig. 14A) direct the foreign gene of interest into the vaccinia virus tk-gene of the novel fowlpox virus host strains (Fig. 7). The abbreviation P2mx stands for the mutated P2 promoters as described in Fig. 15. These insertion plasmids are suited for the high level expression of open reading frames that lack their own translational initiation and termination



codons. The translational stop codons that terminate translation in all three reading frames are provided by the plasmids. An additional feature of the multiple cloning sites of the insertion plasmids pP2m0gpt, pP2m1gpt, pP2m2gpt is a transcriptional stop signal, that terminates poxvirus early gene expression; the sequence of the multiple cloning site is shown in Fig. 14B.

The plasmid pFSgpt (Fig. 13A) also directs the foreign gene of interest into the vaccinia virus tk-gene of the novel fowlpox virus host strains (Fig. 7). It may be used for the cloning of poxvirus-promoter foreign gene cassettes. The plasmid pFSgpt also provides translational stop codons and the poxvirus early transcription stop signal. The sequence of the multiple cloning site is shown in Fig. 13B.

The plasmids pTZgpt-sPx (Fig. 17B) are "promoter test plasmids", which were constructed to test various synthetic promoters (here designated as sPx). The abbreviation sPx may have the following meaning:

- a) sP11 = synthetic VV P11 promoter mutant;
- b) s4b = synthetic FPV 4b promoter mutant;
- c) sart = synthetic promoter mutant.

The above promoters comprise strong late promoters which are active in VV as well as FPV. These promoters may be excised with or without reporter gene (lacZ) and can thus be cloned into various vector systems. These additional promoters enlarge the available promoter pool and allow multiple expression. They also have the advantage that the regions which are homologous to the viral genome are restricted to rather short sequences, a fact that reduces the probability of recombinations and thus decreases instability of recombinant viruses.

As described above, a recombinant FPV which is used for the expression of foreign protein(s) is obtained by in vivo homologous recombination.

The invention comprises also a method for the expression of a foreign protein. This method consists of infecting appropriate host cells with a recombinant FPV according to the invention. The host cells are then cultured to allow expression of the desired protein, and the latter is recovered by means of conventional techniques.

Suitable cells or cell cultures are chicken embryo fibroblast cells or chicken dermal fibroblast cells.

Any desired protein may be expressed by use of the above recombinant FPV and obtained in sufficient quantities. It is of special interest to express proteins which require post-translational modification in a way as it is performed by the host cell. Such proteins are e.g. Factors II, V, VII, VIII, IX, X, XI, XII, XIII, protein C, protein S, von Willebrand-Factor, plasminogen and derivatives thereof, wherein one or more amino acid is replaced, deleted or inserted, partial sequences and activated forms thereof, apolipoproteins, such as apoA1 and apoAII, and viral antigens such as hepatitis B-antigens, the antigens of hepatitis C-virus, the antigens of hepatitis E-virus, the antigens of tick-borne encephalitis (TBE) virus, the antigens of HIV, HSV and whole or partial sequences of such antigens which cause pertussis, tetanus, malaria, poultry diseases, Marek's disease, ILT, infectious, bronchitis, coccidiosis and Newcastle disease, the above antigens being useful as vaccines.

## Experimental Part

### 1. Methods

#### 1.1 Virus and cells

The fowlpox virus strain HPI (Mayr & Malicki; Zentralblatt f. Veterinärmedizin, Reihe B. 13. 1-12 (1966)) and the attenuated strain HPI-441 (passage number 441 of HPI) were kindly provided by Prof. A. Mayr, Munich. Primary chicken embryo fibroblasts (CEF) were prepared as described in the European patent application publication No. 0 338 807. The cells were grown in tissue culture medium 199 (TCM 199; Gibco BRL) supplemented with 5 % fetal calf serum, glutamine and antibiotics. Vaccinia virus (ATCC # VR 119, strain WR) was kindly provided by Dr. B. Moss. The virus was replicated in CV-1 cells and purified as reported by Mackett et al (in D.M. Glover (ed). (1985), DNA cloning: A practical approach; IRL Press, Oxford). The African Green Monkey kidney cell line CV-1 (ATCC # CCL 70) was obtained from the American Type Culture Collection, Rockville, Maryland.

#### 1.2 Purification of fowlpox virus (FPV)

The purification was essentially done as described by Joklik (Virology, 18, 9-18 (1962)) with the following modifications: CEF monolayers (twenty 175 cm<sup>2</sup> cell culture flasks) were infected with 1 pfu(plaque

forming unit/cell and incubated for 4-5 days at +37 °C and 5 %/CO<sub>2</sub>. The cells were scraped into the medium, spun down at 2,000 rpm for 20 minutes in a H6000A-rotor of a Sorvall RC3C centrifuge. The pellet was resuspended in 5 ml of 10 mM Tris pH 9, sonicated, supplemented with 1/10 volume of 2.5 % trypsin and incubated at +37 °C for 30 minutes. To pellet the extracellular virus, the supernatant was centrifuged at 17,000 rpm for 2 hours at +4 °C in a Beckman type 19 rotor. The trypsinized cells and the virus pellet of the cell culture supernatant were pooled, loaded on a 36 % sucrose cushion and centrifuged for 80 minutes at 13,500 rpm in a Beckman SW28 rotor at +4 °C. The pellet was resuspended in 1 ml of 1 mM Tris pH 9, sonicated, layered onto a 20-40 % sucrose gradient and centrifuged at 12,000 rpm for 50 minutes at +4 °C. The two viral bands (the intra- and extracellular forms of the virus) were collected, pooled and 2 volumes of 10 mM Tris pH 9 were added. The viral pellet was collected after centrifugation at 15,500 rpm for 60 minutes and resuspended in 500 µl 1 mM Tris 1 mM NaCl pH 9.

### 1.3 Cell infection and plaque assays

The plaque assays were performed on confluent monolayers of CEFs (in tissue culture dishes; 60 cm<sup>2</sup>, approximately 6x10<sup>6</sup> cells, or in 6 well plates, 10 cm<sup>2</sup>, 1x10<sup>6</sup> cells per well) or CV-1 cells in 6 well plates (10 cm<sup>2</sup>; 1x10<sup>6</sup> cells per well) respectively. The virus suspension was allowed to adsorb to the cells in a volume of 0.6 ml TCM 199 with occasional rocking for 1 hour. The suspension was removed by aspiration and replaced by an overlay consisting of serumfree DMEM, antibiotics and 1 % low melting agarose (LMA; Gibco BRL). FPV-plaques titrated on CEF-cells were stained with 30 µg/ml of neutral red (Sigma) on the 5th or 6th day of infection. Vaccinia virus plaques titrated on CV-1 cells were stained with 50 µg/ml of neutral red on the 3rd day of infection.

### 1.4 In vivo recombination

CEF cells or CV-1 cells (in 60 cm<sup>2</sup> tissue culture dishes) were infected with 1 plaque forming unit (pfu) per cell of HPI-441 or VV, respectively. The virus was adsorbed for 1 hour at +37 °C in 2.5 ml TCM 199. Subsequently the medium was aspirated and the infected monolayers were overlaid with a DNA-Ca-phosphate precipitate, consisting of 20 µg of plasmid DNA and 5 µg of HPI-441 or VV wild-type DNA in Hepes buffered saline in a final volume of 1 ml according to Graham & van der Eb (Virology, 52, 456-467 (1973)). After a 30 minutes incubation period at room temperature 9 ml of TCM 199 were added and the incubation was continued for another 4 hours at +37 °C. The medium was replaced with 10 ml of fresh TCM 199 and the plates were incubated for 2 days. Then the cells were scraped into the medium and the pellets were lysed by three successive cycles of freezing and thawing. Progeny virus was then assayed for the presence of recombinants.

### 1.5 Selection and plaque purification of the recombinants

#### 1.5.1 Blue plaque screening

Viruses with lacZ gene inserts were identified by blue plaque screening as described by Chakrabarti et al (Mol. Cell. Biol., 5, 3403-3409 (1985)) with the following modifications: CEF-cells (in 60 cm<sup>2</sup> tissue culture dishes) or CV-1 cells (in 6 well plates) were infected with viral crude stocks derived from recombination experiments and overlaid with serumfree DMEM containing 1 % LMA. After 5-6 days for CEF and 3 days for CV-1 the monolayers were stained with a second overlay consisting of 1 % LMA in phosphate buffered saline (PBS) and 600 µg/ml of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). Blue plaques appeared about 4-12 hours later.

#### 1.5.2 gpt-selection

Recombinant FPV viruses with gpt-gene inserts were identified on the basis of their resistance to the drug mycophenolic acid (MPA) essentially as described by Falkner & Moss (J. Virol., 62, 1849-1854 (1988)) with the following modifications: monolayers of CEF cells were infected with recombinant virus and overlaid with DMEM supplemented with 125 µg/ml xanthine, 5-25 µg/ml MPA and 1 % LMA. After 5-6 days the plaques were visualized by staining with a second overlay consisting of 1 % LMA in PBS containing 30 µg/ml neutral red. In case of gpt- and lacZ positive recombinants the overlay contained in addition 600 µg/ml of X-gal. The plaques were subjected to several rounds of plaque purification.

Monolayers of CV-1 cells were infected with recombinant vaccinia virus and overlaid with DMEM supplemented with 250 µg/ml xanthine, 15 µg/ml hypoxanthine, 25 µg/ml MPA and 1 % LMA. After 2-3 days the plaques were visualized by staining with a second overlay consisting of 1 % MPA in PBS containing 50 µg/ml neutral red and 600 µg/ml X-gal. The plaques were subjected to several rounds of plaque purification.

## 1.6 Transient expression assays

The assay was essentially done as described by Cochran et al (Proc. Natl. Acad. Sci. USA, 82, 19-23 (1985)) and modified as follows: Confluent monolayers of CV-1 cells (about  $1 \times 10^7$  cells) were infected with 5 or 10 plaque forming units of vaccinia wild-type virus and transfected with 30 µg plasmid DNA in the form of a DNA-Ca-precipitate prepared according to Graham & van der Eb (Virology, 52, 456-467 (1973)). Cells were harvested 24 hours post-infection by centrifugation and resuspended in 100 µl PBS. Cytoplasmic extracts of infected cells were prepared by sonication and assayed for β-galactosidase activity.

## 1.7 β-galactosidase assays

Confluent monolayers of CV-1 cells ( $8 \times 10^6$  cells) were infected with 10 plaque forming units of vaccinia recombinants, harvested 24 hours post-infection by centrifugation and resuspended in 100 µl PBS. For the preparation of cytoplasmic extracts the cells were disrupted by three repeated cycles of freezing and thawing and by sonication. Protein extracts were quantified according to Bradford (Anal. Biochem., 72, 248-254 (1976)). The enzymatic assays were carried out essentially as described by Miller (in Experiments in molecular genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York: 352-355 (1972)) and modified as follows: All reagents were prewarmed to 28 °C, lysates were kept on ice. The reactions were carried out in 770 µl lxZ buffer (0.6 M  $\text{Na}_2\text{HPO}_4$ , 0.4 M  $\text{NaH}_2\text{PO}_4$ , 0.1 M KCl, 0.01 M  $\text{MgSO}_4$ , 0.5 M β-mercaptoethanol, pH 7). 200 µl of the chromogenic substrate o-nitrophenyl-β-D-galactopyranoside (ONPG; 4 mg/ml in 0.1 M phosphate buffer pH 7.0) was added and the reaction was started by adding of 30 µl of diluted (1:100) cell extract. After 3 minutes at room temperature the assay was transferred to a Beckman DU8 photometer. Optical density was recorded at 420 nm for 15 minutes at 28 °C with reference to a PBS sample. Results were confirmed by scanning of polyacrylamide gels using a UV-Vis densitometer (Hirschmann).

## 1.8 Sequencing

The sequences were determined with the T7 polymerase sequencing kit (Pharmacia) by the dideoxy chain termination method (Sanger & Coulson; J. Mol. Biol., 94, 441 (1975)) with specific primers. The construction of the plasmids was performed according to standard techniques as described by Sambrook et al (Molecular cloning; Cold Spring Harbor Laboratory Press (1989)).

## 2. Construction of the insertion plasmids

### 2.1 pFPtk5

As a first step, the FPV thymidine kinase gene was cloned as follows: an EcoRI digest of fowlpox virus DNA (strain HPI Munich) was cloned into the EcoRI site of the vector pTZ19R (Pharmacia). The tk-gene containing plasmid (designated pFPtk5) was identified by colony filter hybridization using the oligonucleotide probe 5'-CAG TTA TTG TGG CCG CGC TTA ACG GTG A-3'. The plasmid contained a 5.5 kb EcoRI fragment.

### 2.2 pFPtk10.4

pFPtk5 was cleaved with ClaI, BamHI and Scal, treated with Klenow polymerase and ligated with the vector pTZ19R, which had been treated with PvuII, EcoRI and phosphatase. The resulting plasmid, pFPtk10.4, had the 2.48 kb BamHI-ClaI insert that contains the fowlpox virus tk-gene (Boyle et al; Virology, 156, 355-356 (1987)).

### 2.3 pFP-UV2i

Into the unique NcoI site within the tk-coding region of pFPtkl0.4, the 2.3 kb SspI fragment from pUVI (Falkner et al; Nucl. Acids Res., 15, 7192 (1987)) was inserted; the fragment contains the PII-promoter (Bertholet et al; Proc. Natl. Acad. Sci. USA, 82, 2096-2100 (1985)), the P7.5 promoter (Cochran et al; J. Virol., 54, 30-37 (1985)) and the 5'-part of the lacZ gene.

### 2.4 pFP-UV2

The cloning of the plasmid pFP-UV2 was completed by inserting the 2.3 kb lacZ fragment (3' part of the lacZ gene) into the intermediate plasmid pFP-UV2i.

### 2.5 pFP-UV2-PT

In the following experiment the cDNA sequence for prothrombin was cloned into plasmid pFP-UV2. This experiment was carried out by excising the 2.0 kb EcoRI fragment from the plasmid pPt # 12, which is described in European patent application No. 90 101 623.8. The complete human prothrombin cDNA was then cloned into the EcoRI and phosphatase treated vector pFP-UV2. In this construct the translational start codon of the prothrombin cDNA is exactly fused with the naturally occurring start codon of the promoter of the vaccinia virus major late IIL polypeptide. The resulting plasmid was designated pFP-UV2-PT.

### 2.6 pTKm

This plasmid was constructed from pFPtkl0.4 by oligonucleotide directed mutagenesis using a phosphorothioate-based mutagenesis procedure (Amersham, Inc.). The mutagenic primer used to enlarge and modify the intergenic region of the FPV thymidine kinase gene had the sequence

5'-TTA CAC TAA  
ACC GGT ACC CGG GAT CGA TAA AAA CCT TAA TTA CTA-3'.

The structure of the mutation was confirmed by sequencing using the primer the 5'-CCATTCCGTGTATAATGTAC-3' located 46 bp downstream of the altered sequence.

### 2.7 pFP-ZsPII

In the plasmid pFP-Z2I (see 2.15), the lacZ gene is flanked by several restriction sites but does not contain promoter sequences. Into the PstI and SmaI sites of pFP-Z2I a synthetic promoter (a modified version of the vaccinia PII promoter) was inserted upstream of the lacZ gene by ligation of a synthetic linker consisting of the annealed oligonucleotides I and II.

(oligonucleotide I, 5'-GCC TAT TTA TAG CAT  
AGA AAA AAA CAA AAT GAA ATT TTA CTA TAT TTT TAT ATA  
CAT ATA TTC TAA CCC-3'; oligonucleotide II, 5'-GGG  
TTA GAA TAT ATG TAT GTA AAA ATA TAG TAG AAT TTC ATT  
TTG TTT TTT TCT ATG CTA TAA ATA GGC TGC A-3').

### 2.8 pTKm-sPII

The 3.3 kb SmaI/BalI fragment, encompassing the E.coli lacZ gene regulated by the synthetic vaccinia late promoter was prepared from the plasmid pFP-ZsPII and inserted into the vector pTKm linearized with SmaI. The resulting plasmid was designated pTKm-sPII.

## 2.9 pTKm-sP11-gpt

pTKm-sP11 was linearized with SmaI and ligated with the 1.1 kb Hpa I-Dra I P7.5-gpt gene cassette excised from the plasmid pTKgpt-Fls (Falkner & Moss; J. Virol., 62, 1849-1854 (1988)). The resulting  
 5 plasmid was designated pTKm-sP11-gpt.

## 2.10 pTKm-VVtka and b

These plasmids were constructed by inserting the complete vaccinia virus thymidine kinase gene,  
 10 prepared as a 1.1 b Dra I fragment from pGS50 (Mackett & Smith; J. Gen. Virol., 67, 2067-2082 (1986)) into the SmaI linearized vector pTKm-sP11. The resulting plasmids were designated pTKm-VVtka and b.

## 2.11 MI3mpl8-UVI

As a first step, the 1.2 kb PstI/SauI fragment derived from the insertion vector pFP-UV2 (see 2.4) was  
 15 subcloned into MI3mpl8. This fragment encompasses the promoters of the vaccinia virus genes coding for the I1K (P11; Bertholet et al; Proc. Natl. Acad. Sci. USA, 82, 2096-2100 (1985)) and 7.5K polypeptides (P7.5; Cochran et al; J. Virol., 54, 30-37 (1985)) and part of the lacZ gene. The resulting plasmid was designated  
 20 MI3mpl8-UVI.

## 2.12 MI3mpl8-Eco2

Oligonucleotide directed mutagenesis (Amersham, Inc.) was used to introduce a second EcoRI site 7 bp  
 upstream the ATG of the lacZ gene of MI3mpl8-UVI, creating the intermediate plasmid MI3mpl8-Eco2. The  
 25 mutagenic primer used to alter the lacZ upstream region had the sequence 5'-ACC ATA TGT AAG GAA TTC CTT AGA TAA-3'.

## 2.13 pFP-UV2-Eco2

The modified PstI/SauI promoter fragment prepared from MI3mpl8-Eco2 was inserted into PstI/SauI cut  
 30 pFP-UV2 and the resulting vector was designated pFP-UV-2-Eco2.

## 2.14 pFP-ZI

The plasmid pFP-ZI was constructed by deleting the 0.9 kb EcoRI P11/P7.5 fragment from pFP-UV2-  
 35 Eco2, thereby placing the multiple cloning site immediately upstream the lacZ gene.

## 2.15 pFP-Z2I

The plasmid pFP-Z2I was constructed by introducing a synthetic linker sequence (5'-CGA TTG GCC  
 40 AGG ATC CGT CGA CAG GCC TAT-3'; complementary strand, 5'-CGA TAG GCC TGT CGA CGG ATC CTG GCC AAT-3') into the partially ClaI digested vector pFP-ZI. This modification allows the simple excision of the lacZ gene.

## 45 2.16 pFP-2

The plasmid pFP-2 was isolated from a library constructed by inserting random fragments of  
 SspI/EcoRV digested FPV-DNA (HPI-441) into the plasmid pFP-ZI linearized with SmaI.

## 50 2.17 pFP-ZP2

The 0.6 kb EcoRI/NsiI fragment containing the P2 promoter activity was prepared from pFP-2. This  
 fragment was ligated with the EcoRI/PstI linearized vector pFP-Z2I.

## 55 2.18 pTZgpt-P2a and pTZgpt-P2b

These plasmids were constructed by inserting the P2-lacZ gene cassette derived from pFP-ZP2 (a 3.7  
 kb SmaI/StuI fragment) into the HpaI linearized plasmid pTZgpt-dP (see 2.27). The resulting vectors were

designated pTZgpt-P2a and pTZgpt-P2b.

## 2.19 pFS50

- 5 In a first step, the plasmid pTZ19R (Pharmacia) was digested with PvuII to delete a 349 bp fragment containing the multiple cloning site and adjacent sequences. This vector fragment was ligated with a 1.1 kb vaccinia tk-gene fragment prepared from pGS50 by DraI digestion. The resulting plasmid was designated pFS50.

## 10 2.20 pFS5I

pFS50 was cut with ClaI and EcoRI and ligated with a synthetic linker (P-MCS1 and 2). This vector was designated pFS5I. The oligonucleotides used for linker construction had the sequence: P-MCS1,

15

5'-CGA

GCA GCTG CAT ATG AGG CCT GGA TCC CGG GTC GAC GCG GCC  
GCT AAC TGA CTG ATT TTT CTC-3' and P-MCS2, 5'-AAT TGA  
20 GAA AAA TCA GTC AGT TAG CGG CCG CGT CGA CCC GGG ATC  
CAG GCC TCA TAT GCA GCT GCT-3'.

25

## 2.21 pFSgpt

- The plasmid pFSgpt was generated by subcloning a 0.98 kb P7.5-gpt gene cassette, prepared from pTKgpt-Fls (Falkner & Moss; J. Virol., 62, 1849-1854 (1988)) by digestion with NdeI and DraI, into the  
30 PvuII/NdeI cut plasmid pFS5I.

## 2.22 pP2mOgpt

- Synthetic oligonucleotides encoding the mutant mO P2 promoter were annealed and inserted by forced  
35 cloning into the NdeI/BamHI linearized vector pFSgpt. The nucleotide sequences of these oligonucleotides are as follows: m0.1:

5'-TAC GGC TTG GTA TAG CGG ACA ACT AAG

40

TAA TTG TAA AGA AGA AAA CGA AAC TAT CAA AAC CGT TTA  
TGA AAT GAT AGA AAA AAG AAT ATA AAT AAT CCT GTA TTT  
TAG TTT AAG TAA CAG TAA AAT AAT GAG TAG AAA ATA CTA  
45 TTT TTT ATA GCC TAT AAA TGA ATT CG-3'. m0.2: 5'-GATC  
CGA ATT CAT TTA TAG GCT ATA AAA AAT AGT ATT TTC TAC  
TCA TTA TTT TAC TGT TAC TTA AAC TAA AAT ACA GGA TTA  
TTT ATA TTC TTT TTT CTA TCA TTT CAT AAA CGG TTT TGA  
50 TAG TTT CGT TTT CTT CTT TAC AAT TAC TTA GTT GTC CGC  
TAT ACC AAG CCG-3'.

- 55 The resulting plasmid was designated pp2mOgpt.

## 2.23 pP2mlgpt

To construct pP2mlgpt the synthetic linker sequences m1.1 and m1.2 were annealed and ligated with the NdeI/BamHI linearized vector pFSgpt. The oligonucleotides had the following sequences: m1.1:

5'-TAC GGC TTG GTA TAG CGG ACA ACT AAG TAA TTG TAA  
AGA AGA AAA CGA AAC TAT CAA AAC CGT TTA TGA AAT GAT  
AGA AAA AAG AAT ATA AAT AAT CCT GTA TTT TAG TTT AAG  
TAA CAG TAA AAT AAT GAG TAG AAA ATA CTA TTT TTT ATA  
GCC TAT AAA TCA TGA ATT CG-3'. m1.2: 5'-GAT CCG AAT  
TCA TGA TTT ATA GGC TAT AAA AAA TAG TAT TTT CTA CTC  
ATT ATT TTA CTG TTA CTT AAA CTA AAA TAC AGG ATT ATT  
TAT ATT CTT TTT TCT ATC ATT TCA TAA ACG GTT TTG ATA  
GTT TCG TTT TCT TCT TTA CAA TTA CTT AGT TGT CCG CTA  
TAC CAA GCC G-3'.

The resulting plasmid was designated pP2mlgpt.

## 2.24 pP2m2gpt

The vector pP2m2gpt was created by ligation of annealed oligonucleotides m2.1 and m2.2 with the NdeI/BamHI cut plasmid pFSgpt. The oligonucleotides used for cloning had the following sequences: m2.1:

5'-TAC GGC TTG GTA TAG CGG ACA ACT AAG TAA TTG TAA  
AGA AGA AAA CGA AAC TAT CAA AAC CGT TTA TGA AAT GAT  
AGA AAA AAG AAT ATA AAT AAT CCT GTA TTT TAG TTT AAG  
TAA CAG TAA AAT AAT GAG TAG AAA ATA CTA TTT TGT TTT  
ATA GCC TAT AAA TCA TGA ATT CG-3'. m2.2: 5'-GATC CGA  
ATT CAT GAT TTA TAG GCT ATA AAA CAA AAT AGT ATT TTC  
TAC TCA TTA TTT TAC TGT TAC TTA AAC TAA AAT ACA GGA  
TTA TTT ATA TTC TTT TTT CTA TCA TTT CAT AAA CGG TTT  
TGA TAG TTT CGT TTT CTT CTT TAC AAT TAC TTA GTT GTC  
CGC TAT ACC AAG CCG-3'.

The resulting plasmid was designated pP2m2gpt.

## 2.25 pP2mOgpt-lacZ, pP2mlgpt-lacZ and pP2m2gpt-Lacz /pP2mxgpt-lacZ)

The construction of pP2mOgpt-lacZ, pP2mlgpt-lacZ and pP2m2gpt-lacZ was done by inserting the E.coli lacZ gene as a 3.2 kb EcoRI/BamI fragment (derived from plasmid pFP-Z2I) into the EcoRI/SmaI linearized vectors pP2mOgpt, pP2mlgpt and pP2m2gpt, respectively.

## 2.26 pTZgpt-Fls

The vaccinia virus insertion vector pTZgpt-Fls was constructed by replacing the 2.4 kb PvuII fragment (originally derived from the plasmid pUC 18) of pTKgpt-Fls (Falkner & Moss; J. Virol., 62, 1849-1854 (1988))

by the 2.5 kb PvuII fragment from the plasmid pTZ19R (Pharmacia, Inc.). In addition to the ampicillin resistance gene and the plasmid origin of replication (also present on the 2.4 kb pUC PvuII fragment) the bacteriophage fl origin of replication was introduced into pTKgpt-Fls by this cloning step.

## 5 2.27 pTZgpt-dP

The PII promoter of pTZgpt-Fls was deleted by digestion of PstI and HpaI and the large vector fragment ligated with HpaI linkers (5'-GGTTAACC-3', Pharmacia Inc.). The resulting plasmid was designated pTZgpt-dP.

10

## 2.28 MI3mpl8-UV3

The plasmid MI3mpl8-UV3 was constructed by oligonucleotide directed in vitro mutagenesis (Pharmacia, Inc.) of the PII promoter in the vector MI3mpl8-UVI (see 2.11). The oligonucleotide used to alter the promoter region had the sequence: 5'-TAGCTATAA ATAAAGAATT CCTGCAG-3'-.

15

## 2.29 pTZgpt-PIIM

The vaccinia virus recombination plasmid pTZgpt-PIIM was constructed by inserting a 600 bp HindIII/Asp718 Klenow polymerase treated fragment derived from MI3mpl8-UV3 into the HpaI digested pTZgpt-dP plasmid. This fragment contains the mutated PII promoter (PIIM).

20

## 2.30 pFP-ZsPII

The oligonucleotides sPII(3) and sPII(4) were annealed and cloned into the SmaI/PstI cut vector pFP-Z2I (see 2.15). The sequences of sPII(3) and of sPII(4) were

25

5'-GCCTATTTAT AGCATAGAAA AAAACAAAAT  
GAAATTCTAC TATATTTTTA CATAATATA TTCTAACCC-3' and  
5'-GGGTTAGAAT ATATGTATGT AAAAATATAG TAGAATTTCA  
TTTTGTTTTT TTCTATGCTA TAAATAGGCT GCA-3',

30

35

respectively. The resulting plasmid was designated pFP-ZsPII.

## 2.31 pTZgpt-sPII

pFP-ZsPII was digested with SmaI/BalI and the 3.3 kb fragment containing the synthetic promoter sequence linked to the lacZ gene was cloned into the vaccinia virus insertion vector pTZgpt-dP (see 2.27). The resulting plasmid was designated pTZgpt-sPII.

40

## 2.32 pFP-Zs4b

45

The oligonucleotides s4b(3) and s4b(4) were annealed and cloned into the SmaI/PstI cut vector pFP-Z2I (see 2.15). The sequences of s4b(3) and of s4b(4) were

5'-GCCTATTTAT ATTTGATAGT TTTTACTTG TAACGTATCA  
AAATAAGTAC CTAAAGAGAC CTAACCCC-3' and 5'-GGGGTTACGT  
CTCTTTAGGT ACTTATTTTG ATACGTTACA AGTAAAAAAC  
TATCAAATAT AAATAGGCTG CA-3',

50

55

respectively. The resulting plasmid was designated pFP-Zs4b.



## 2.33 pTZgpt-s4b

pFP-Zs4b was digested with SmaI/BalI and the 3.3 kb fragment containing the synthetic promoter sequence linked to the lacZ gene was cloned into the vaccinia virus insertion vector pTZgpt-dP (see 2.27).

5 The resulting plasmid was designated pTZgpt-s4b.

## 2.34 pFP-Zsart

The oligonucleotides sart(3) and sart(4) were annealed and cloned into the SmaI/PstI cut vector pFP-Z2I  
10 (see 2.15). The sequences of sart(3) and sart(4) were

5' -GCCTATTTAT ATGCCAAAAA AAAAAAAAAA  
AAAAAGCTTC CC-3' and 5' -GGGAAGCTTT TTTTTTTTTT  
15 TTTTTTTGGC ATATAAATAG GCTGCA-3',

respectively. The resulting plasmid was designated pFP-Zsart.

## 20 2.35 pTZgpt-sart

pFP-Zsart was digested with SmaI/BalI and the 3.3 kb fragment containing the synthetic promoter sequence linked to the lacZ gene was cloned into the vaccinia virus insertion vector pTZgpt-dP (see 2.27). The resulting plasmid was designated pTZgpt-sart.

25 Recombinant viruses vflsβ, vPII, vPIIm, v4b and vart were derived from recombination plasmids pTKgptFIβ (Falkner & Moss; J. Virol., 62, 1849-1854 (1988)), pTZgpt-sPII, pPIIm-lacZ (T. Langmann, Diplomarbeit, Universität Wien 1991), pTZgpt-s4b and pTZgpt-sart, respectively.

### 3. Relevance of the fowlpox virus thymidine kinase gene for growth in the cell culture

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#### 3.1 Construction of the FPV insertion plasmid pFP-UV2 and pFP-UV2-PT

In the first type of plasmids constructed, pFP-UV2 and pFP-UV2-PT, the coding sequence of the fowlpox virus tk-gene is split into two fragments by the foreign gene inserts. The plasmid pFP-UV2 has a  
35 similar structure as the vaccinia virus insertion plasmid pUVI (Falkner et al; Nucl. Acids Res., 15, 7192 (1987)). In pUVI the E.coli lacZ reporter gene is driven by the vaccinia virus early/late p7.5 promoter (Cochran et al; J. Virol., 54, 30-37 (1985)). The promoter of the vaccinia virus major late I1K polypeptide (Bertholet et al; Proc. Natl. Acad. Sci. USA, 82, 2096-2100 (1985)) is followed by the multiple cloning site and serves as the regulatory element of the foreign gene to be inserted. Both components are flanked by  
40 sequences of the vaccinia tk-gene (Fig. 1A). The plasmid pFP-UV2 has the same arrangement of the lacZ reporter gene and the promoters. It is flanked, however, by fowlpox virus tk-gene sequences (Fig. 1A). Insertion of pFP-UV2 into the genomic tk-locus of FPV by in vivo recombination will, as in the case of pUVI, result in the inactivation of the viral tk-gene. To construct this plasmid, the promoter-lacZ gene cassette of pUVI was cloned in two steps into the unique NcoI site within the FPV tk-gene as outlined in Fig. 1A. To  
45 construct the recombination plasmid pFP-UV2-PT the human prothrombin cDNA was inserted into the vector pFP-UV2 downstream of the vaccinia I1K promoter. The plasmid pFP-UV2-PT was used for the construction of the first series of FPV recombinants.

#### 3.2 Genomic characterization of FPV recombinants derived from pFP-UV2-PT

50

In order to investigate the functional properties of the plasmid pFP-UV2-PT, an in vivo recombination experiment in chicken embryo fibroblasts was carried out. Due to the lacZ reporter gene, recombinant virus could be identified by blue plaque screening. Several blue plaques were picked and plaque purified three times. Southern hybridizations of total DNA from cells infected with the FPV recombinants with the FPV tk-  
55 gene and the lacZ gene probes, however, revealed that the predicted banding pattern of the restriction fragments (one novel band of about 8.3 kb with the lacZ gene and two bands of about 1.9 and 8.3 kb with the FPV tk-gene probe) could not be observed. Instead a complex pattern of bands was seen, including the wild-type tk-gene band. One typical viral isolate, designated f-PTI blue, was chosen for further rounds of

plaque purification and Southern analysis. Beginning with the three times plaque purified isolate, a total of 11 rounds of plaque purification did not change the complex banding pattern significantly. In lanes 1-5 of Fig. 2A and 2B, the Southern analysis of viral DNAs of the 3rd, 5th, 7th, 9th and 11th round of plaque purification as visualized with the FPV tk-gene probe (Fig. 2A) and the lacZ gene probe (Fig. 2B) is shown. Another isolate, f-PT2 blue, showed a similar but not identical DNA banding pattern (Fig. 2A and 2B, lane 6).

During all rounds of plaque purification frequently white plaques were observed. Two white plaques, f-PT1 and 2 white, were analyzed together with the f-PT blue isolates. They hybridized with the lacZ gene probe but failed to develop the blue colour (Fig. 2B, lanes 8 and 9). Hybridization with the FPV tk-gene probe revealed in all cases the presence of the wild-type tk-gene (Fig. 2A, lanes 1 to 9, arrowhead). The FPV wild-type control is seen in lane 7 and the negative control (chicken embryo fibroblast DNA) in lane 10. To demonstrate that these unexpected findings were not due to a partial EcoRI digest the same blot as shown in Fig. 2A was hybridized to a prothrombin gene probe. As shown in Fig. 2C in all cases (except in lane 6; these larger bands may be due to a more complex recombination event in the f-PT2 blue genome) only one band, the 2.0 kb prothrombin band, was detected, indicating that the restriction digest was complete. The complex bands seen in Fig. 2A and 2B are therefore not artifacts due to a partial digest of the viral DNA, but reflect that the inactivation of the viral tk-gene with the insertion plasmid pFP-UV2-PT a genomically monoclonal fowlpox recombinant could not be obtained.

### 3.3 Construction of the FPV insertion plasmid pTKm-sPIL-gpt

In order to check the hypothesis, that the tk-gene is essential in FPV strain HPI.441, a novel insertion plasmid, pTKm, was constructed. The site of insertion of a foreign gene in this plasmid is located in the intergenic region between the tk-gene and the open reading frame downstream of the tk-gene (3'orf). The wild-type intergenic region between the tk-gene and the 3'orf does not contain unique restriction sites for inserting foreign genes. This region was therefore modified as shown in Fig. 3. Immediately downstream of the stop codon of the tk-gene a vaccinia early transcription stop signal (Rohrmann G. et al, Cell 46: 1029 (1986)) and the unique restriction sites ClaI, SmaI and Asp718 were introduced by site directed mutagenesis. Into the modified enlarged intergenic region of the plasmid pTKm the synthetic PII promoter-lacZ gene cassette and the P7.5-gpt gene cassette were inserted in two steps. The plasmid finally obtained was designated pTKm-sPIL-gpt (Fig. 4); it contains the lacZ gene for blue plaque screening and the gpt-gene as a selective marker. Upon integration into the viral genome, it will not split and inactivate the FPV tk-gene.

### 3.4 Recombinant fowlpox viruses with intact FPV tk-gene

The plasmid pTKm-sPIL-gpt was subsequently used to construct FPV recombinants by in vivo recombination in chicken embryo fibroblasts. All plaques obtained under gpt-selection also stained blue in the presence of X-gal in the overlay. Only two rounds of plaque purification (under gpt-selection) were necessary to obtain the viral isolates.

Subsequently, one of the isolates, designated f-sPIL#1, was grown to large scale and purified. Viral DNA of the recombinant and of wild-type virus was digested with EcoRI, separated on an agarose gel and analyzed by Southern blotting. In Fig. 5A-C different probes were used; the individual lanes represent the following:

- A) Hybridization with the FPV tk-gene probe. Lane 1 represents DNA of FPV recombinant f-sPIL#1, lane 2 represents DNA of the FPV wild type virus HPI.441; lane 3 represents lambda DNA digested with HindIII.
- B) Hybridization with the gpt-gene probe. Lane 1 represents lambda DNA digested with HindIII; lane 2 is DNA of FPV recombinant f-sPIL#1; lane 3 is DNA of the FPV wild type virus HP 1.441.
- C) Hybridization with the lacZ gene and phage lambda DNA probes. Lane 1 represents lambda DNA digested with HindIII; lane 2 is DNA of the FPV recombinant f-sPIL#1; lane 3 is DNA of the FPV wild type virus HPI.441. The values given on the right for comparison correspond to standards in kilo basepairs.

In Fig. 5A the restriction fragments were hybridized with the FPV tk-gene probe. In the recombinant DNA two novel fragments of 5.2 kb and 4.7 kb are visible (lane 1); in the control DNA the 5.5 kb wild-type tk-band can be seen. After hybridization with the gpt-gene probe (Fig. 5B), the 5.2 kb fragment that contains part of the tk-gene and the gpt sequences lights up (lane 2) while with the wild-type virus (lane 3) no signal is obtained. Finally, hybridization with the lacZ gene and the phage lambda probes (Fig. 5C) reveals the 4.7 kb lacZ gene containing fragment of the recombinant virus (lane 2) and the marker bands (lane 1). Again the wild-type virus (lane 3) does not hybridize.

It is concluded from this experiment that the intergenic region between the FPV tk-gene and the 3'orf is non-essential and that an intact tk-gene allows the purification of legitimate FPV recombinants.

### 3.5 Novel FPV host strains: f-TK2a and F-TK2b

5

For technical and biological reasons it is more difficult and more time-consuming to construct recombinant FPV. Therefore, prior to inserting a gene of interest into FPV, a similar vaccinia virus recombinant was usually constructed to study the function of the respective gene. To be able to use the same vaccinia insertion plasmids also for the construction of fowlpox recombinants, the vaccinia virus tk-gene, together with the E.coli lacZ gene was inserted into the intergenic region of the tk-gene and the 3'orf of fowlpox virus. The plasmids pTKm-VVtka and pTKm-VVtkb were constructed by cloning the functional VV tk-gene into the intermediate plasmid pTKm-sP11 (Fig. 4). Upon recombination of pTKm-VVtka and b with FPV-wild-type virus two novel FPV host strain (termed f-TK2a and f-TK2b) were created. The novel host strain thus contains two functional tk-genes and the lacZ gene, all of which can be used as novel non-essential sites with the appropriate insertion plasmids as recombination substrates. The Southern blot analysis of the novel strains is shown in Fig. 6A-C. The DNA's of the wild type virus HPI.441, the two FPV recombinants and the plasmids pTKm-VVtka and pTKm-VVtkb were digested with the restriction enzymes PstI, ClaI and EcoRI, separated on a 1 % agarose gel, transferred onto nitrocellulose. The blots in Figs. 6A-C were hybridized with the FPV-tk-gene probe (6A), with the vaccinia virus tk-gene probe (Fig. 6B) and with the lacZ gene and the lambda DNA probes (Fig. 6C). In all digests the predicted banding pattern (see also Figs. 6D and 6E) could be observed. In case of the ClaI digests the small hybridizing fragments of about 0.5 and 0.7 kb in lanes 5 and 6 of Fig. 6B cannot be seen in the ClaI digested parental plasmids (lanes 13 and 14 of Fig. 6B). This is due to the fact that the plasmid DNAs were isolated from the E. coli strain HB101, a strain that methylates the respective ClaI site. In a control experiment this site was cleavable when the DNA was prepared from a Dam methylation negative E. coli strain.

## 4. Fowlpox virus early/late promoter in vaccinia virus recombinants

### 4.1 Identification of the fowlpox promoters

30

A transcription unit consisting of a vaccinia virus promoter and the coding sequence of the lacZ gene is active in E.coli cells resultings in a  $\beta$ -galactosidase positive phenotype of the bacterial colonies grown in the presence of the chromogenic substrate X-gal. This phenomenon has been observed when working with vaccinia insertion plasmids containing the E.coli lacZ gene in lacZ negative E.coli strains (Chakrabarti et al; Mol. Cell. Biol., 5, 3403-3409 (1985)). Since the promoter sequences of FPV and vaccinia are functionally equivalent (Boyle & Coupar; J. Gen. Virol., 67, 1591-1600 (1986); Taylor et al; Vaccine, 6, 497-503 (1988)) also fowlpox promoters should be active in E.coli. Based on these considerations a strategy was worked out for the identification of promoter elements in fowlpox virus DNA.

As the first step the plasmids pFP-ZI and pFP-Z2I were constructed (Fig. 8); both plasmids contain a promoterless lacZ gene. As the parental plasmid, pFP-UV2 was chosen. It contains the E.coli lacZ gene controlled by the vaccinia virus P 7.5 promoter, the P11 promoter and a multiple cloning site for cloning purposes and is flanked by fowlpox virus tk-sequences. In order to delete the vaccinia promoters a novel EcoRI site was introduced 7 bp upstream of the initiation codon of the lacZ gene. Cleavage with EcoRI and religation resulted in the plasmid pFP-ZI that contains unique restriction sites adjacent to the promoterless lacZ gene. In the next step the DNA of the fowlpox virus strain HPI-441 was digested with the restriction endonucleases SspI and EcoRV and cloned into the unique SmaI site adjacent to the lacZ gene of the plasmid pFP-ZI (Fig. 9). The plasmids were transfected into the  $\beta$ -galactosidase negative E.coli strain NM 522 and plated onto agar-dishes containing ampicillin and X-gal. After overnight growth, a small percentage of the colonies developed a blue color. Several colonies were picked and the plasmid DNAs were assayed in a transient expression assay in CV-1 cells for vaccinia virus specific gene expression (data not shown). The plasmid DNAs induced varying amounts of  $\beta$ -galactosidase activity in the vaccinia transient expression assay. For further analysis the clone that gave the highest activity (clone #2) was chosen, the promoter was designated "P2" and the plasmid pFP-2 (Fig. 9).

### 4.2 Structure of the fowlpox virus promoter P2

The DNA of the 2.5 kb P2 promoter insert of the plasmid pFP-2 was analyzed by restriction mapping. The 560 bp EcoRI-NsiI fragment was found to be proximal to the lacZ gene and would therefore contain the

promoter sequences. This fragment was inserted into the plasmid pFP-Z2I, a derivative of pFP-ZI that has at the 3' end of the lacZ gene a polylinker insert (Fig. 8). The promoter lacZ gene cassette was then excised and cloned into the single HpaI site of the plasmid pTZgpt-dP resulting in the plasmids pTZgpt-P2a and pTZgpt-P2b (Fig. 9). Since the orientation of the promoter foreign gene transcription unit may influence the level of transcription both plasmids were used for further investigation. Sequencing of the promoter insert was carried out using the plasmid pTZgpt-P2a as the template. The primary structure of the promoter and the first ten codons of the P2 gene is shown in Fig. 10A. The 5' untranslated region is 174 bp long starting with a NsiI site. Upstream of the initiation codon the conserved poxvirus promoter consensus sequence TAAAT is present (Fig. 10A, pos. -6 to -2) that is typical for late promoters, but is also found in some early promoters. Within the first 174 bp of the upstream region also several "critical early regions" followed by an early transcription stop signal (TTTTNT) are present. The early transcription stop signal overlaps with the functional important T-rich region of the late promoters.

The P2-lacZ transcription unit in the plasmid pTZgpt-P2a was a fusion gene. The initiation codon was followed by 360 bp of the P2-gene fused in frame with 39 nucleotides of the 5' untranslated region and the coding region lacZ gene (data not shown). The calculated molecular weight of the fusion gene is 133 kD.

#### 4.3 Comparison of the strength of the P2 promoter with other pox virus promoters

The plasmids pTZgpt-P2a and pTZgpt-P2b were used for the construction of the vaccinia virus recombinants vP2a and vP2b. The strength of the P2 promoter in both recombinants was compared with other strong pox virus promoters. The vaccinia recombinant vFIs $\beta$  (Falkner & Moss; J. Virol., 62, 1849-1854 (1988)) contains the wild-type version of the vaccinia pII promoter. The vaccinia recombinant vart contains a modified version of the synthetic late promoter that is 1.4 fold stronger than the PII wild-type promoter. In all viruses the lacZ reporter gene is immediately adjacent to the respective pox virus promoters.

For the  $\beta$ -galactosidase activity assays CV-1 cells were infected with the viruses as described in the Methods part. Fig. 11 shows the enzymatic activities induced by the different viral constructs in CV-1 cells. The activity of the wild-type PII promoter in vFIs $\beta$  was defined as 100 %. Remarkably, the "b"-orientation of the FPV P2 promoter induced an activity of 190 %, indicating that the P2 promoter belongs to the strongest pox virus promoters. After a 24 hour incubation period, the  $\beta$ -galactosidase is one of the most abundant proteins and accounts for about 6.3 % of the total soluble cellular proteins. The recombinant virus having the "a"-orientation of the P2 promoter induced a  $\beta$ -galactosidase activity of 150 % which accounts for about 5 % of the soluble cellular protein. The virus vart was found to induce 140 % of  $\beta$ -galactosidase activity as compared to the standard value induced by vFIs $\beta$ . The  $\beta$ -galactosidase activity measurements are mean values of three independent experiments. In order to confirm these values by an independant second method the 24 hour extracts of infected CV-1 cells were separated on 10 % polyacrylamide gels and scanned with a densitometer. The  $\beta$ -galactosidase peaks were quantified relative to the 42 kDa actin band as an internal standard. The value obtained for vFIs $\beta$  again served as the 100 % standard. The scanning data are in good agreement with the enzymatically determined activity data as shown in the following table.

Table

Virus Relative $\beta$ -galactosidase expression (% $\beta$ -galactosidase of soluble protein)		
	% Activity	% Scanning
vFIs $\beta$	100 (3.3%)	100
vP2a	150 (5.0%)	150
vP2b	190 (6.3%)	195
vart	140 (4.6%)	n.d.

To illustrate the abundance of the  $\beta$ -galactosidase in the cell extracts 24 hours post-infection, a commassie blue stained polyacrylamide gel of the total soluble proteins is shown in Fig. 12. The reference virus, vFIs $\beta$ , and the recombinant vart induce a novel band in the 117 kD size range (lanes 3, 4, 9 and 10; lower arrow) that cannot be seen in the wild-type virus control (lanes 1 and 2). As suggested by the sequence analysis, the  $\beta$ -galactosidase fusion protein induced by the viruses vP2a and vP2b is larger than the native enzyme, proving its fusion gene character (lanes 5 to 8; upper arrow).

#### 4.4 Optimization of the P2 promoter

In an attempt to optimize the P2 gene promoter a panel of novel insertion plasmids were constructed, that contain mutated P2 promoter regions linked with the lacZ gene. As the first step a plasmid was generated that allows the simple insertion of double stranded promoter oligonucleotides and contains a minimal P7.5-gpt gene cassette for selection purposes. The construction of this plasmid, pFSgpt, is shown in Fig. 13. Into the unique NdeI and BamHI sites of this plasmid, the different mutant promoter oligonucleotides m0, m1 and m2 were inserted. The resulting plasmids were designated pP2m0gpt, pP2m1gpt and pP2m2gpt (pP2mxgpt, Fig. 14). In the next step the E.coli lacZ gene was placed downstream of the promoter sequences resulting in the plasmids pP2m0gpt-lacZ, pP2m1gpt-lacZ and pP2m2gpt-lacZ (pP2mxgpt-lacZ, Fig. 14).

In the mutant promoter m0 (TAAATG AAT TCC) the ATG of the lacZ gene is directly fused with the late promoter core sequence thereby deleting the C-residue at position -1 of the wild-type P2 sequence, a mutation that should improve the efficiency of a late promoter (Fig. 15). This structure is found in many vaccinia late promoters and is thought to be the optimal context of the late promoter consensus and the initiation codon.

In the mutant m1 (TAAACATG AAT TCC) the second codon of the lacZ gene is directly fused with the ATG of the putative P2 gene. In this mutant the lacZ gene is driven by the P2 wild-type promoter (Fig. 15).

The mutation m2 was constructed in order to investigate the role of the early promoter critical regions found in the upstream region of the P2 gene. The mutant promoter m2 has the same structure as m1 except that the early RNA stop signal within the functional important T-rich region upstream the late promoter motif was inactivated by a TTG insertion at position -18 (Fig. 15).

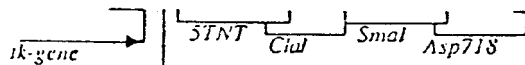
#### 4.5 Effect of the mutations on early and late $\beta$ -gal expression

The plasmids were used to construct vaccinia virus recombinants and to infect CV-1 cells. Cytoplasmic extracts were assayed for  $\beta$ -galactosidase activity. Results are shown in Fig. 16.

#### Claims

1. A plasmid, characterized in that it comprises at least the FPV tk-gene, a downstream intergenic region and the following 3' open reading frame (3' orf) of fowlpox virus (FPV), wherein said intergenic region is modified to form an enlarged intergenic region which comprises one or more unique restriction sites thereby allowing insertion of foreign DNA in such a way that the FPV tk-gene remains intact and codes for the entire thymidine kinase (TK).
2. The plasmid according to claim 1, characterized in that the sequence of the tk-gene is followed by a pox virus early transcription stop signal.
3. The plasmid according to claim 1, characterized in that said enlarged intergenic region comprises the following sequence:

AGTAATTAAGGTTTTATCGATCCCGGGTACCGGTTTAGTGAATAAATTTAATAAAATATTGACAAAATAGT



enlarged intergenic region

TAAATGAATATATGAAAGTACATTATACACGGAAATGGAG



enlarged intergenic region

4. The plasmid according to claim 1, obtainable by site-directed mutagenesis.

5. The plasmid according to claim 3, obtainable by site-directed mutagenesis of the wild-type sequence with the primer sequence  
5'-TTACACTAAATCGGTACCCGGGATCGATAAAACCTTAATTACTA-3'—

- 5 6. Plasmid according to pTKm of Fig. 4A.
7. A plasmid for insertion of foreign DNA into FPV by in vivo homologous recombination, said plasmid being prepared from the plasmid according to claims 1 to 6 and comprising:
- 10 (a) a natural or synthetic poxvirus promoter linked to a foreign DNA sequence which is to be expressed;
- (b) a second pox virus promoter linked to a gene encoding a marker or indicator for selection of recombinant FPV;
- 15 (c) DNA sequences of FPV flanking the construct of elements (a) and (b) at both 5' and 3' ends, said flanking DNA sequences being homologous to the sequences upstream and downstream of the enlarged intergenic region.
8. A plasmid according to claim 7, characterized in that it further comprises a replicon for plasmid replication in a prokaryotic host, and a gene encoding a selectable marker or indicator for selection of the plasmid in a transformed prokaryotic host.
- 20 9. A plasmid according to claims 7 and 8, characterized in that the promoter is immediately adjacent to the coding sequence of the foreign gene.
10. A plasmid according to claims 7 to 9, characterized in that the pox virus promoter is the FPV P2 promoter having a partial or the entire DNA sequence as shown below:
- 25

NsiI -125  
ATGCATTTGT TAGAGCTTGG TATAGCGGAC AACTAAGTAA TTGTAAAGAA  
-99 -95 -86  
30 GAAAACGAAA CTATCAAAAC CGTTTATGAA ATGATAGAAA AAAGAATATA  
-42  
AATAATCCTG TATTTTAGTT TAAGTAACAG TAAAATAATG AGTAGAAAAT  
-19 -1 +1  
35 ACTATTTTTTTATAGCCTATAAATC ATG GAA AAG AAA CTG ATT CAA GAG  
TAT GAA

or a functional equivalent thereof.

40

11. A plasmid according to claims 7 to 10, characterized in that the pox virus promoter is a mutant of FPV promoter P2 having a DNA sequence as shown in Fig. 15.
- 45 12. A plasmid according to claims 7, characterized by insertion plasmid pTKm-sPll-gpt of Fig. 4A, wherein the lacZ gene is replaced by the DNA sequence to be expressed.
13. A recombinant FPV for the expression of a foreign protein or as a vaccine, obtainable by in vivo recombination of wild-type FPV with a plasmid according to claims 7 to 12.
- 50 14. Recombinant FPV f-TK2a and f-TK2b as host virus comprising at least an intact FPV tk-gene as well as a VV tk-gene, whereof either one may serve as non-essential site (NES) for the insertion of one or more foreign DNA sequences.
15. Recombinant FPV according to claim 14, characterized in that it comprises additionally a selection marker and/or a reporter gene.
- 55 16. Plasmid according to pTZgpt-P2a of Fig. 9.

17. Plasmid according to pTZgpt-P2b of Fig. 9.
18. Plasmids according to pP2m0gpt, pP2mlgpt and pP2m2gpt (pp2mxgpt) of Fig. 14A.
- 5 19. Plasmid according to pFS50 of Fig. 13A.
20. Plasmid according to pFS5l of Fig. 13A.
21. Plasmid according to pFSgpt of Fig. 13A.
- 10 22. Plasmid according to pTZgpt-Fls of Fig. 17A.
23. Plasmid according to pTZgpt-PIIM of Fig. 17A.
- 15 24. A plasmid for the insertion of foreign DNA and subsequent integration into the host strains according to claim 14 by in vivo recombination, characterized by one of the following plasmids:
  - a) pFP-UV2 of Fig. 1, pTKm of Fig. 4A, pFS50, pFS5l and pFSgpt of Fig. 13A, pP2mxgpt of Fig. 14A, pTZgpt-Fls and pTZgpt-PIIM of Fig. 17A, and
  - b) pTKm-sPII-gpt of Fig. 4A, pTZgpt-P2a and pTZgpt-P2b of Fig. 9 and pTZgpt-sPx of Fig. 17B, wherein the lacZ gene inserts in these plasmids is replaced by DNA sequences to be expressed.
- 20 25. The plasmid according to claim 24, wherein sPx in the construct of pTZgpt-sPx has the meaning of sPII, s4b, sart, as shown in Fig. 18.
- 25 26. Recombinant FPV for the expression of a foreign protein or as a vaccine, obtainable by in vivo recombination of the host strain according to claim 14 and any of the plasmids according to claim 24.
27. An in vitro culture of vertebrate cells infected with a recombinant FPV according to claim 13 or 26.
- 30 28. An in vitro culture according to claim 27, characterized in that the same consists of chicken embryo fibroblast (CEF) cells or chicken embryo dermal (CED) cells.
29. A process for the recombinant production of a protein, characterized by infecting vertebrate host cells or cell cultures with a recombinant virus according to any of the preceding claims, said recombinant virus comprising DNA coding for said protein, and harvesting said protein.
- 35 30. The process according to claim 29, characterized in that one of the following proteins is expressed: Factors II, V, VII, VIII, IX, X, XI, XII, XIII, protein C, protein S, von Willebrand-Factor, plasminogen and derivatives thereof, wherein one or more amino acid is replaced, deleted or inserted, partial sequences and activated forms thereof, apolipoproteins, such as apoAI and apoAII, and viral antigens such as hepatitis B-antigens, the antigens of hepatitis C-virus, the antigens of hepatitis E-virus, the antigens of tick-borne encephalitis (TBE) virus, the antigens of HIV, HSV and whole or partial sequences of such antigens of organisms which cause pertussis, tetanus, malaria, poultry diseases, Marek's disease, ILT, infectious, bronchitis, coccidiosis and Newcastle disease, the above antigens being useful as vaccines.
- 40 31. Vaccine against antigens of pathogens infecting avians and vertebrates, characterized in that the vaccine comprises a recombinant FPV according to claims 13 to 15 and 26.
- 45 32. DNA sequence of the FPV P2 promoter according to claim 10, a partial sequence thereof or a functional equivalent thereof.
- 50 33. A DNA sequence comprising the FPV P2 promoter, the P2 gene and the 3'-sequence as shown below

*Nsil*

-125

ATGCATTTGT TAGAGCTTGG TATAGCGGAC AACTAAGTAA TTGTAAAGAA GAAACGAAA CTATCAAAAC  
-59 -55 -56 -62  
CGTTTETGAA TGTATGAAA AAGAAATATA AATAATCCTG TATTTTAGTT TAGGTACAG TAAATATATG  
-59 -62  
5 AGTGAABAT ACTATTTTTTATAGCCTATA AATCATGGAA AAGAACTGA TTCAAGAGTA TGAAABACTC  
AAGGCCAAG AGGCCAAGA TGTCTTTACC AGGCAGCTAC TTATCTGCCA CGAAGATATG CGTGGCAGAA  
TGGACAACAT GACTAAGTTA ATTAGTGACG TATTTAGAAC ATTGGCTGGA GGTAGTAGCA AAGCACCAC  
10 CGAGAACTCG GATATTGATA CGATGCCTCC TTCTAATGAT GCTGGTTCTG AGCCACAGCC CCAACCTAGC  
GAAAGTAAAC CACCCGAGCA ACCCTCTCCC GAACCCGAAA AAGACTCTTC TAGTAAACCA TCAGATCAAC  
CTACTCCCGA ACCCGAAAA GGCTCTTCTA GCAACCCCG TACAGATATC TTTAGTGGTT TACGTAATA  
15 AGAATTAAAT TTTTGAAAGA ACTGTTGGAG CATTTATCCA ATATTATTAT TTAATTTAAA TCAATTGAGT  
TAATGTAATA ACTTTTTACA TATATTTTGC TCTAGTCCGA AATAGGAAAT TAGCAAAAA TAATGATTAT  
TATATATTAA TGTTTTAACT TAATAATTAA TTTATAAAAT ATTTATTGTC ACATCCGTTT TTTATCACGT  
20 TATCGTACGT GGTAGGTAGT TATGGATGTT TTTATCATTA CTTTTGTAA TGATAGTAAA TAGTATCACA  
GCAGATTTAA TTACTTCTGT GATATACAAT ACATGTATTA AGGATTACCC GCACCCAAA ATAATATCGT  
GATCTGTAAT ATATAAAAT AAATACCATA CTATATGTTT ATATCACCAG TCATGGTAAA AATAGTGTTT  
25 *EcoRI*  
GTGATGAATTC

or a partial sequence thereof, or a functional equivalent thereof.

30 34. Recombinant FPV, characterized in that the 3'-region downstream of the FPV P2 gene is used as non-essential site for the insertion of foreign DNA.

35

40

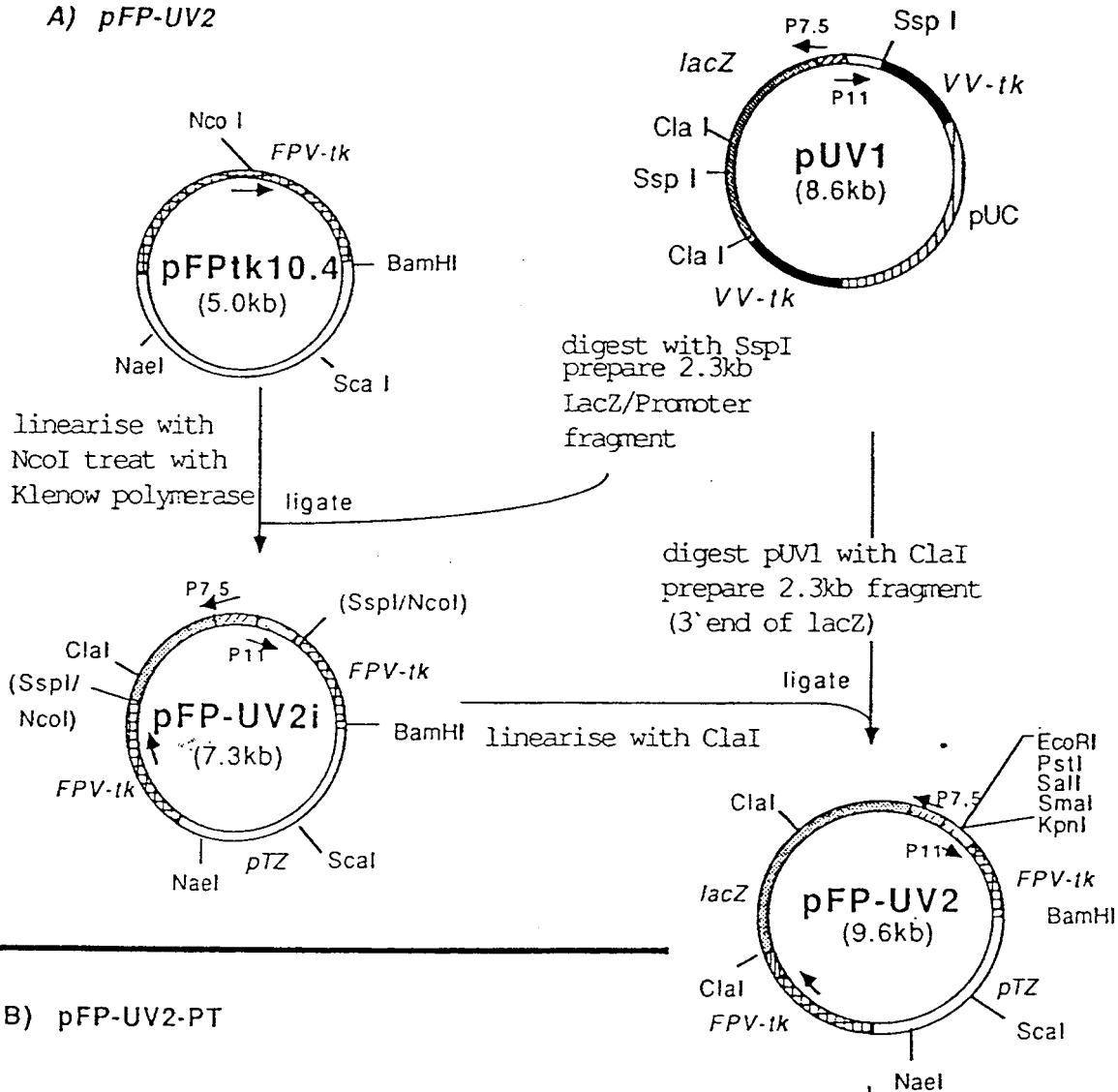
45

50

55



## A) pFP-UV2



## B) pFP-UV2-PT

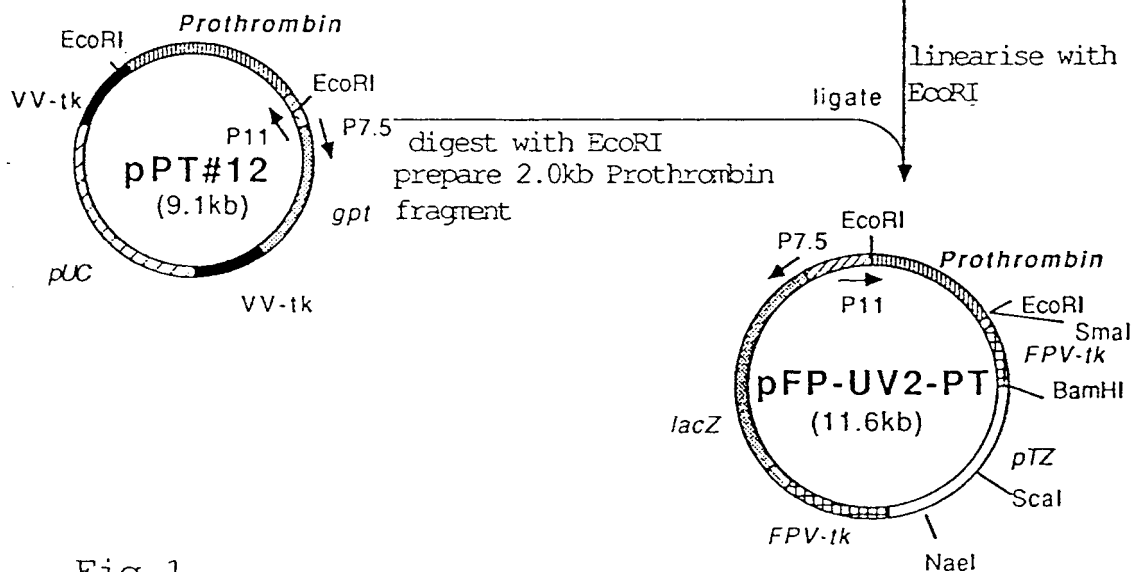


Fig.1

Fig. 2 A

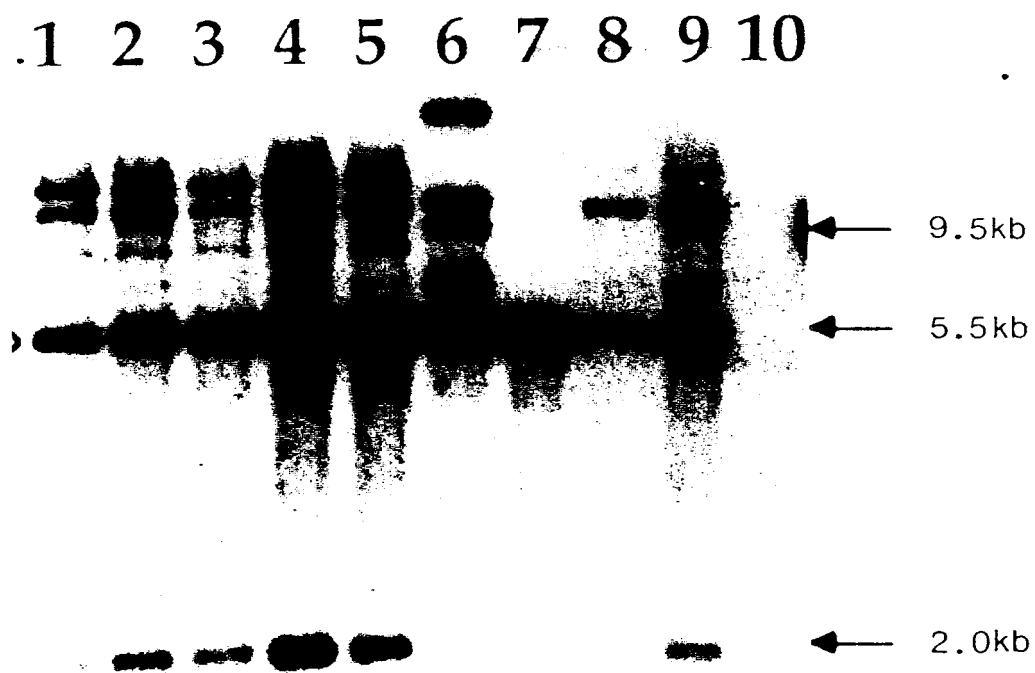


Fig. 2 B

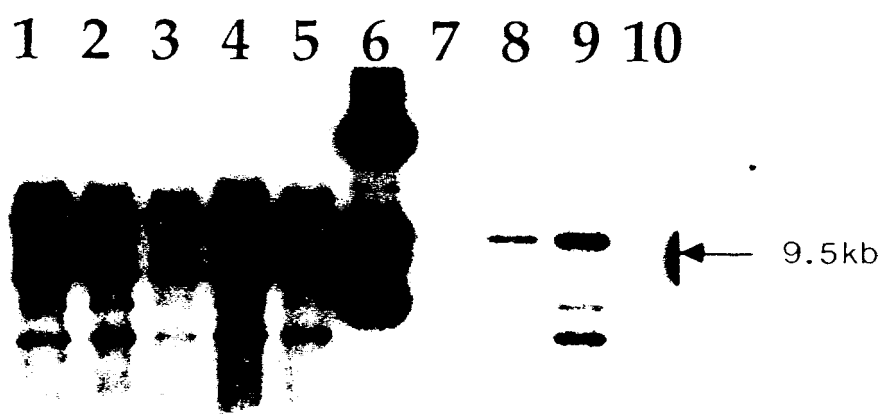


Fig. 2 C

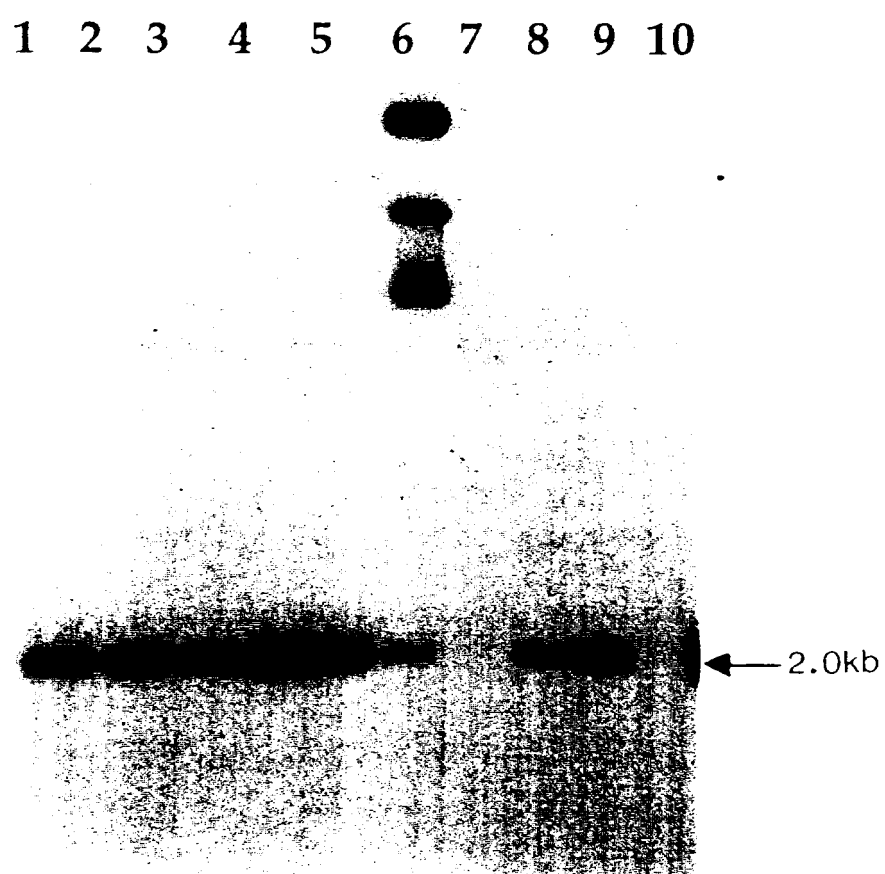


Figure 3A)

### Structure of the FPV-region in the Insertionsplasmid; pTKm

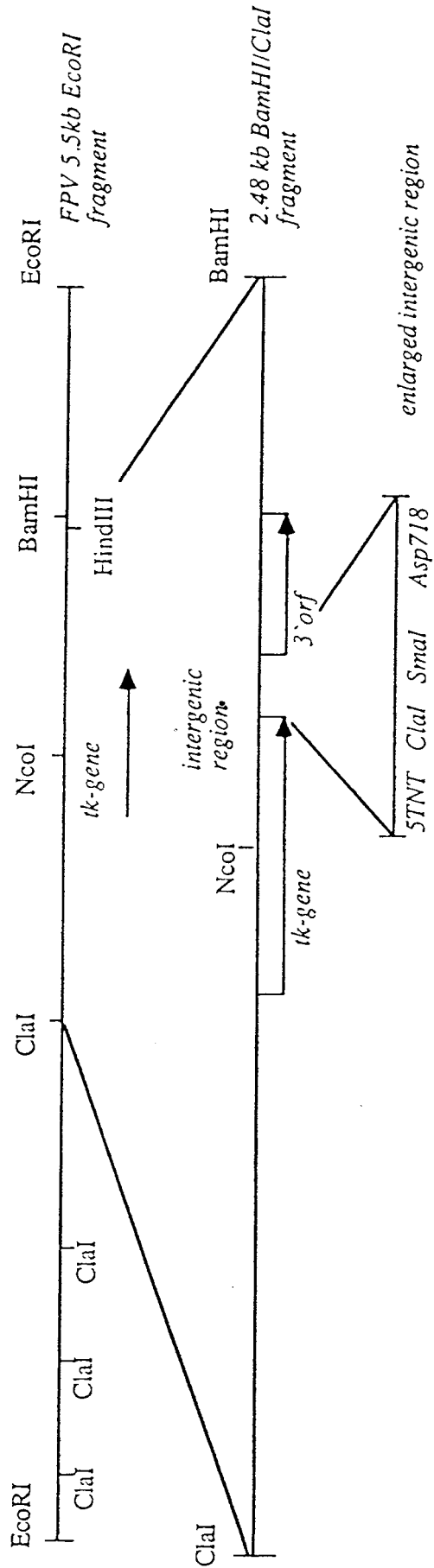
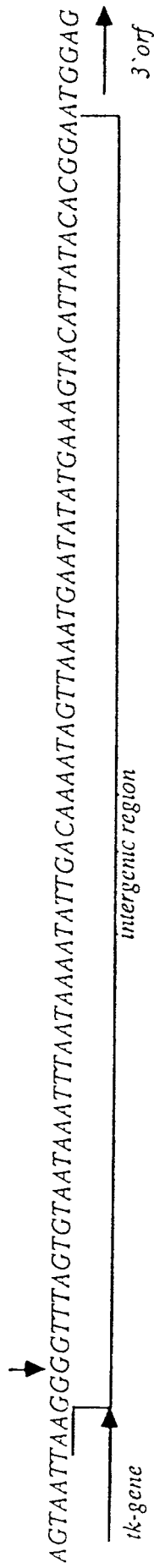


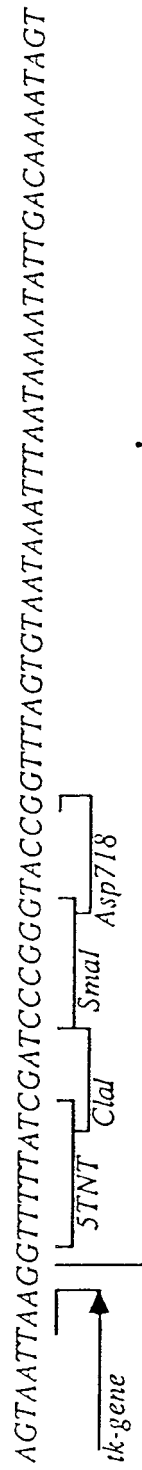
Figure 3B)

*FPV-wildtype intergenic sequence*

site directed  
mutagenesis

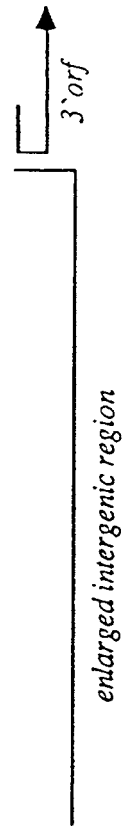


*FPV-enlarged intergenic sequence*



enlarged intergenic region

TAAATGAATATATGAAAGTACATTATACACGGAATGGAG



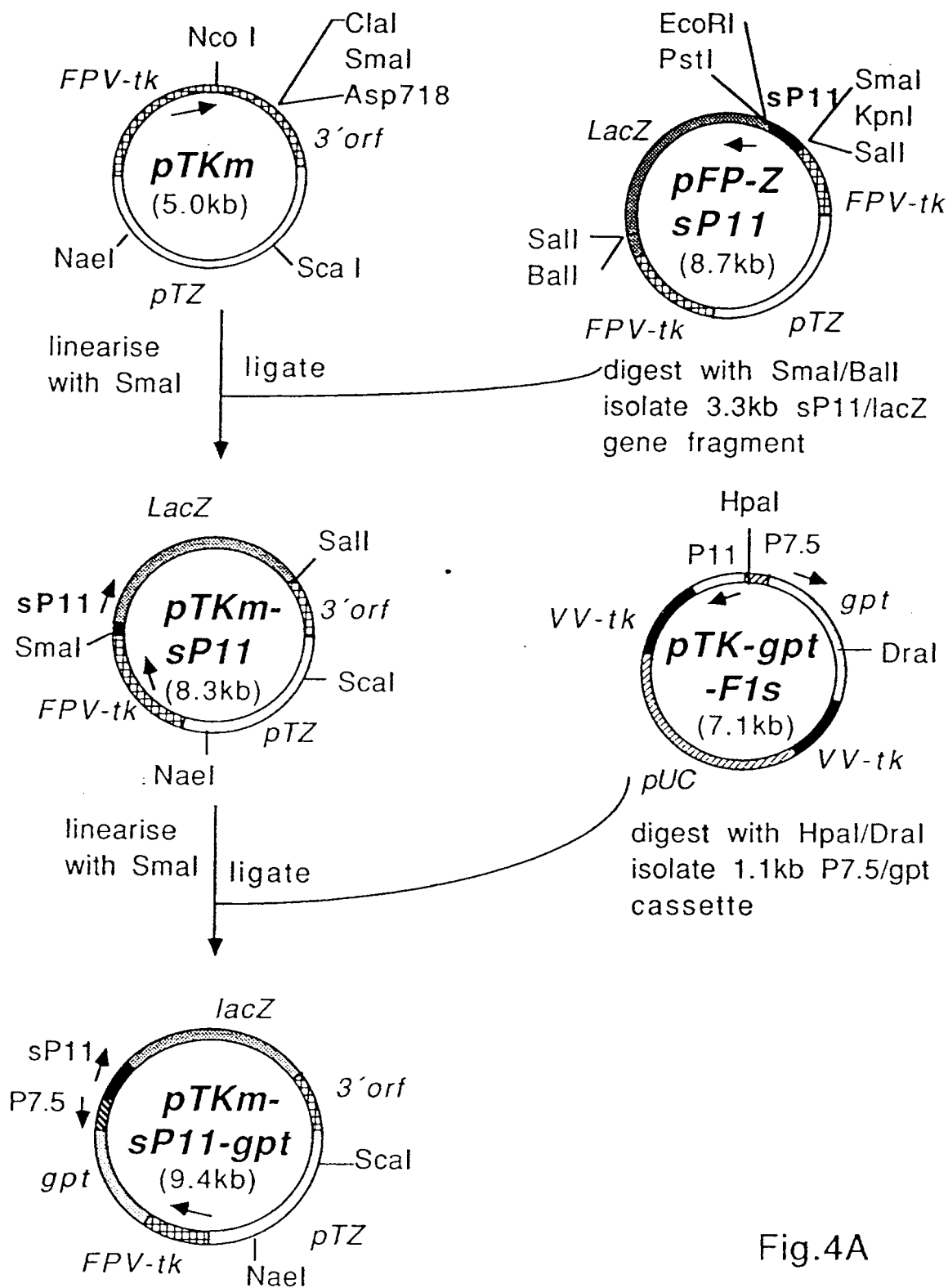


Fig.4A

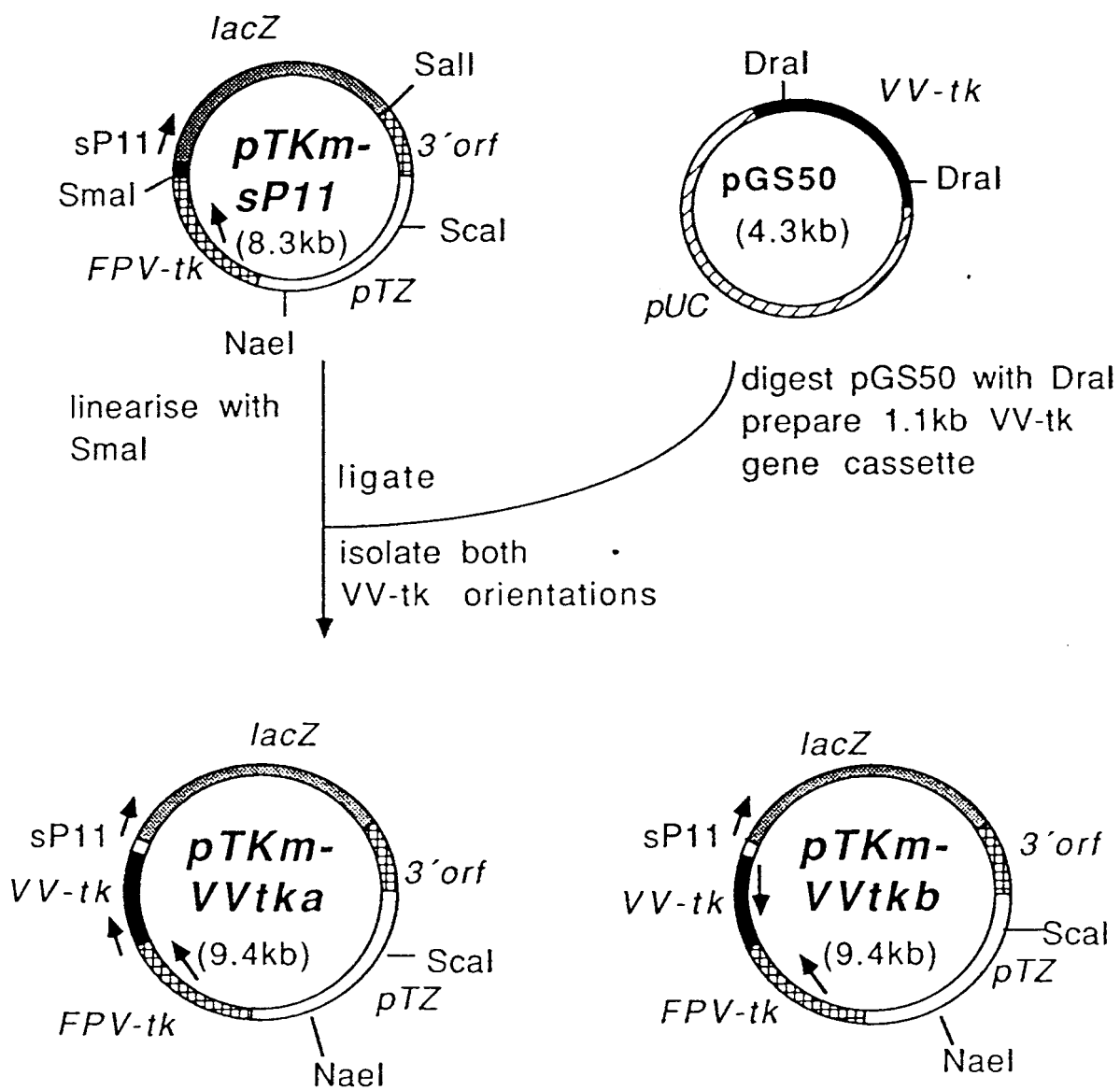


Fig.4B



Fig. 5

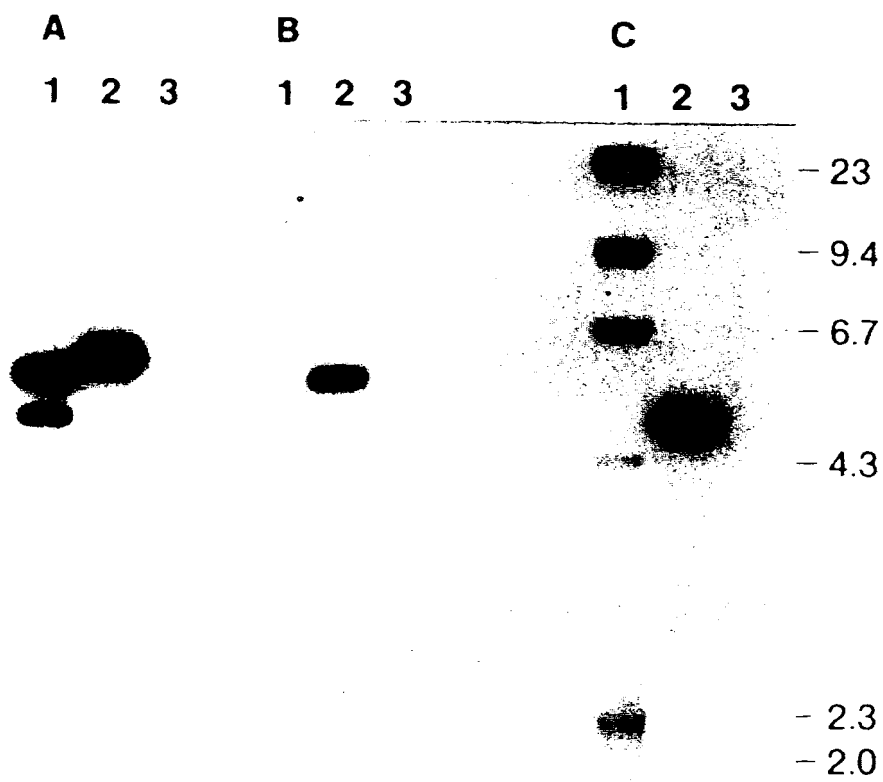


Fig. 6A

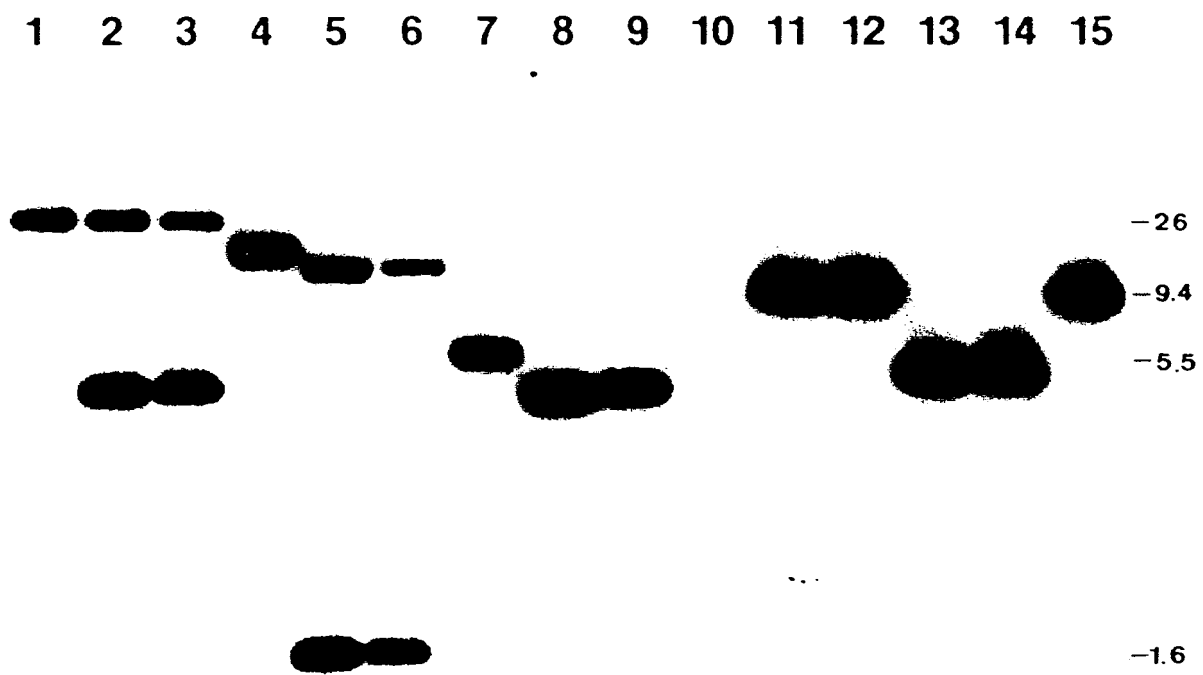


Fig. 6B

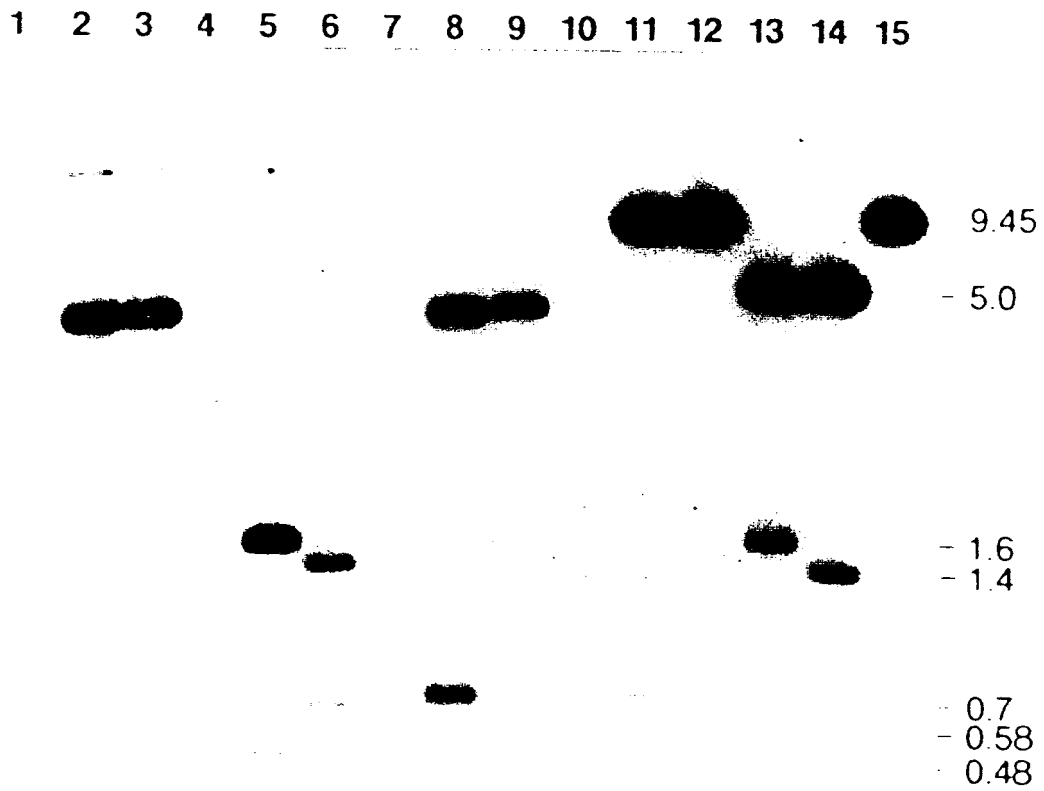
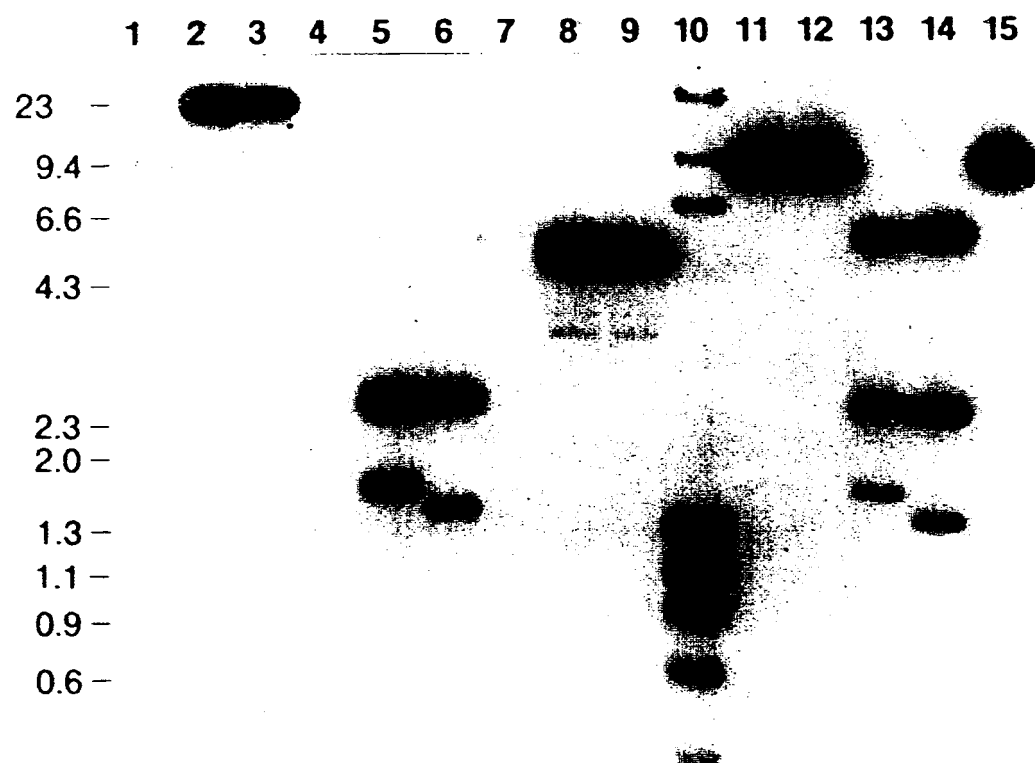


Fig. 6C



Structure of the FPV recombinant f-TK2a

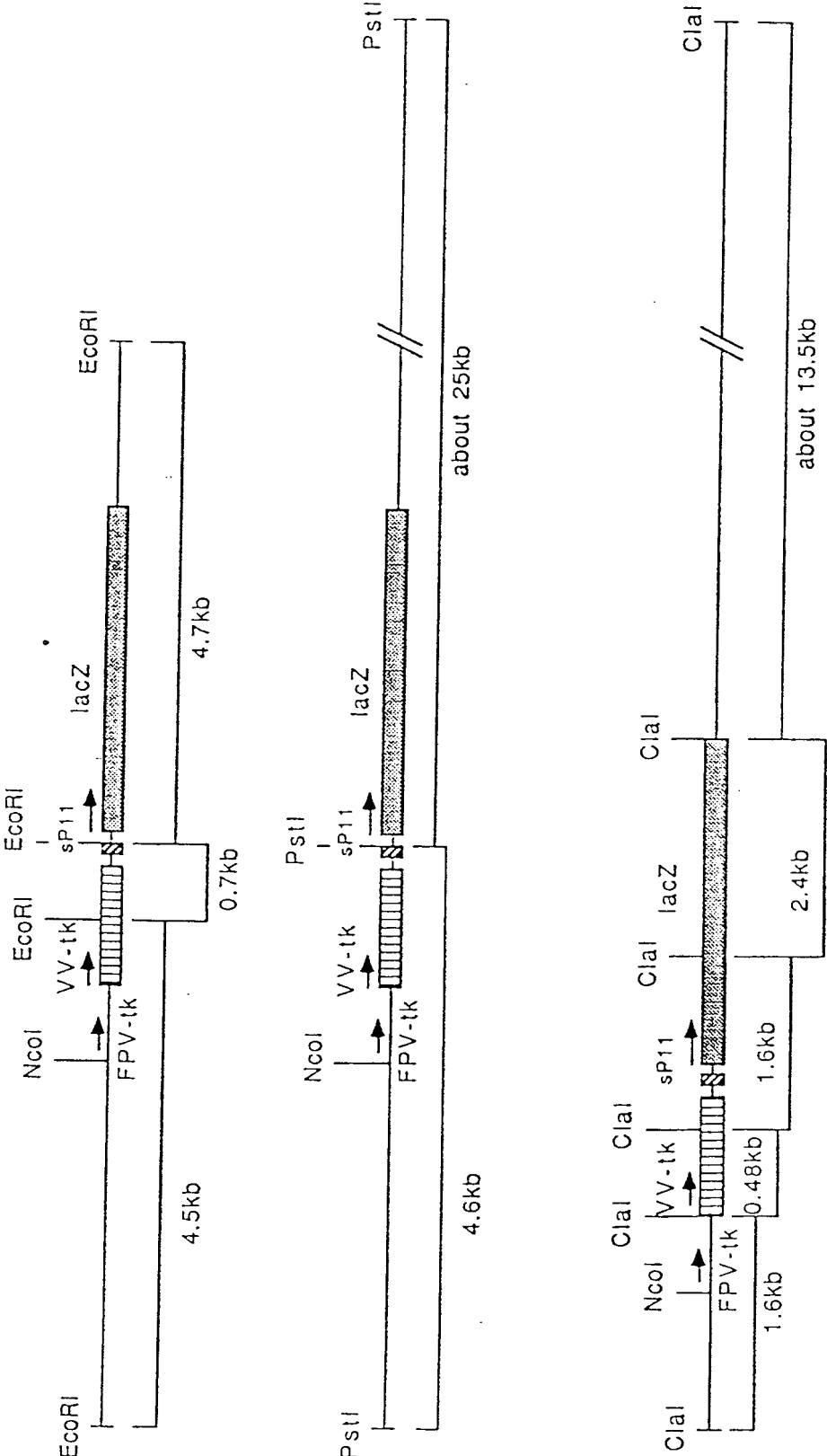


Fig. 6D

# Structure of the FPV recombinant f-TK2b

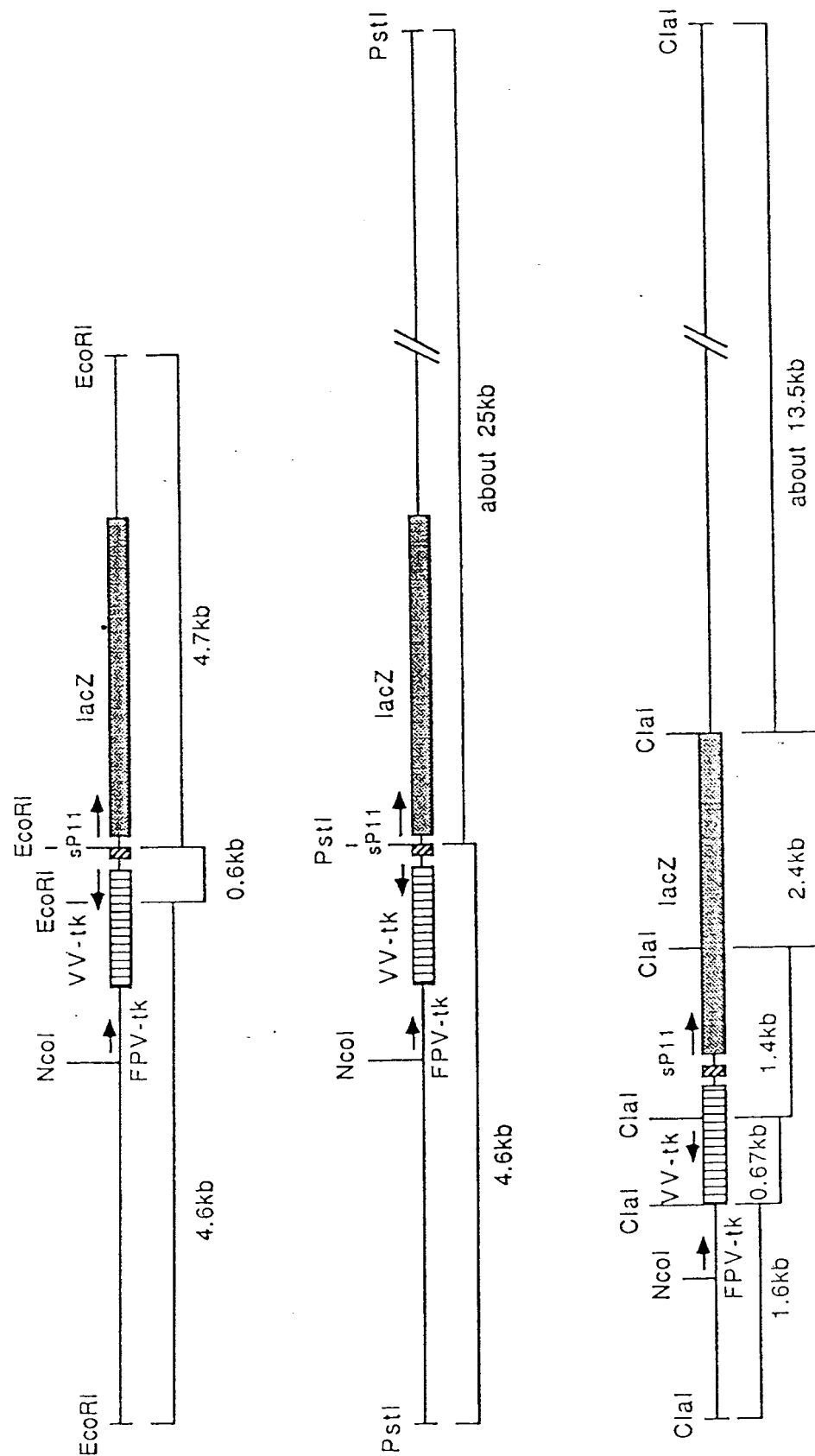


Fig.6E

Structure of the intergenic region of:

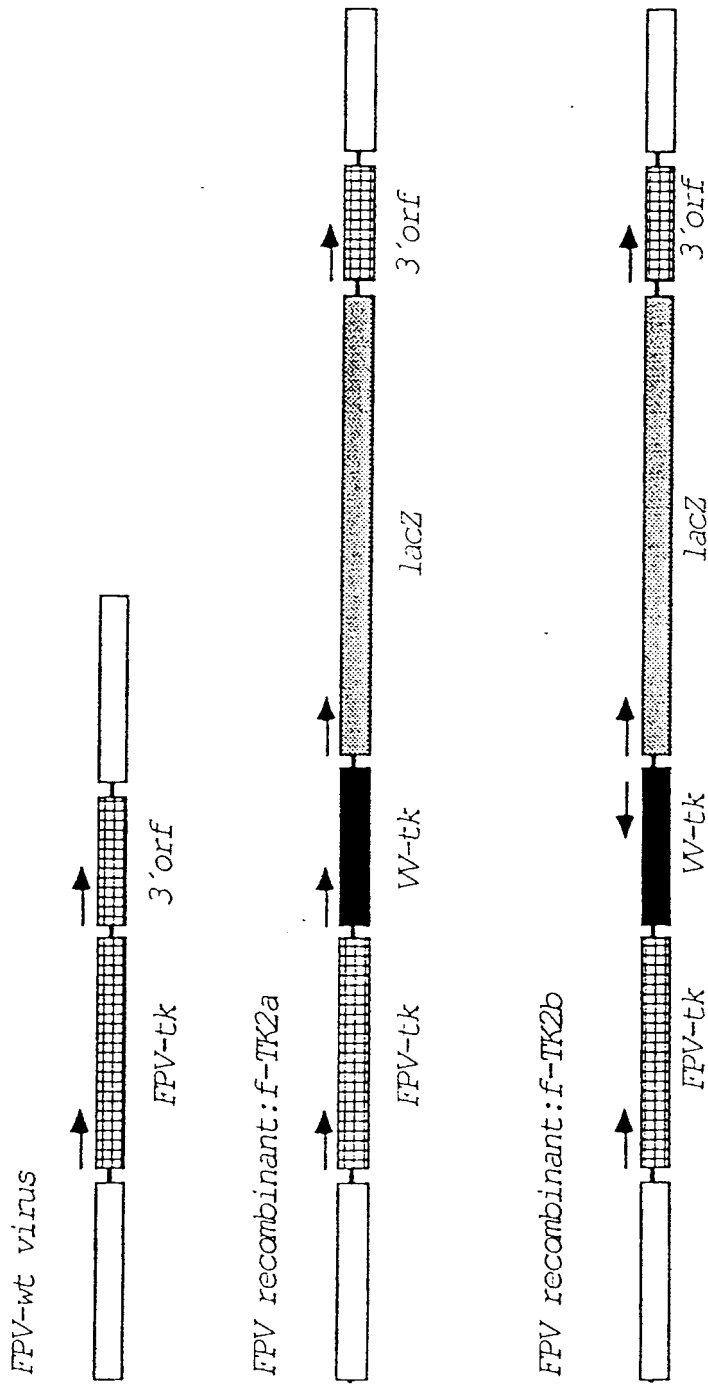


Fig.7

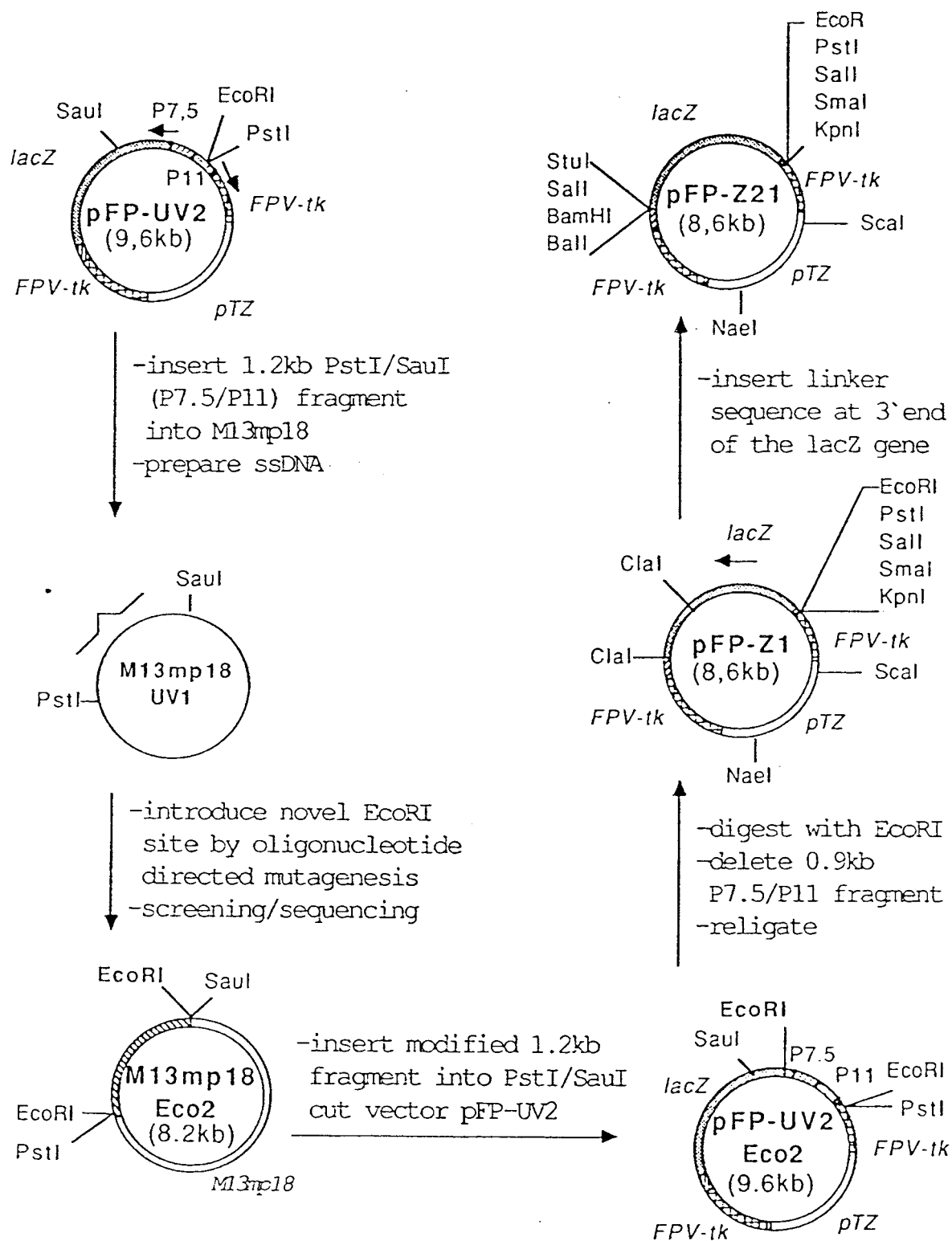
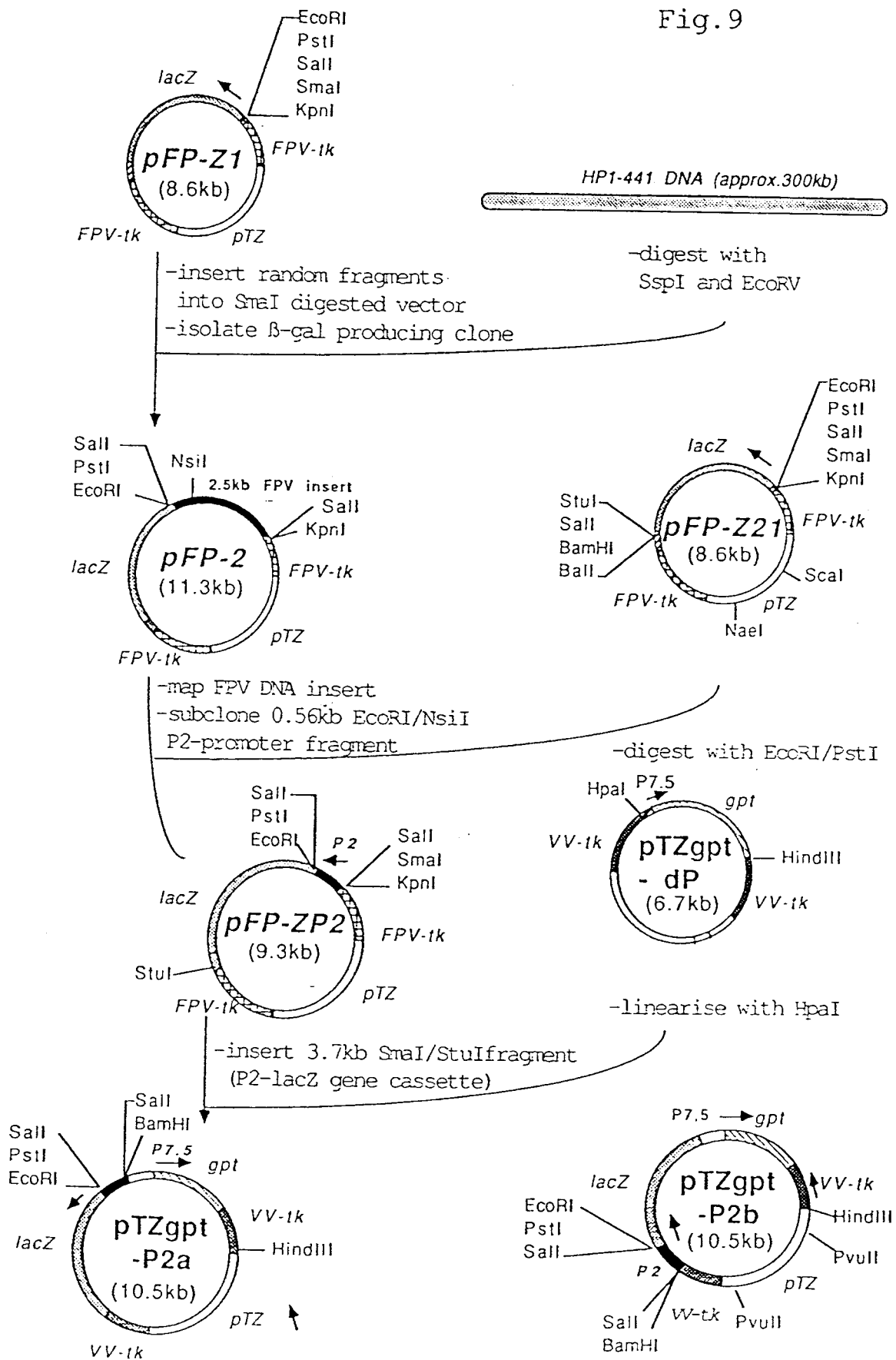


Fig.8



Fig. 9



*Nsi*//  
 -125  
 ATGCATTGT TAGAGCTTGG TATAGCGGAC AACTAAGTAA TTGTAAGAA GAAACGAAA CTATCAAAAC  
 -99 -95 -86 -42  
 CGTTTATGAA ATGATAGAAA AAAGAATATA AATAATCCTG TATTTAGTT TAAGTAACAG TAAAATAATG  
 -19 -1 +1  
 AGTAGAAAAT ACTATTTTATAGCCCTATAATC ATG GAA AAG AAA CTG ATT CAA GAG TAT GAA

Fig.10A

*Nsi*I  
 -99 -95 -86 -19 -1+1 -42 -125  
 ATGCATTGT TAGAGCTTGG TATAGCGGAC AACTAAGTAA TTGTAAAGAA GAAAACGAAA CTATCAAAAC  
 CGTTTATGAA ATGATAGAAA AAAGAATATA AATAATCCTG TATTTTAGTT TAAGTAACAG TAAATAAATG  
 AGTAGAAAAAT ACTATTTTTTATATAGCCTATA AATCATGGAA AAGAAACTGA TTCAAGAGTA TGAANAACCTC  
 AAAGGCCAAG AGG'CAAAGA TGCTTTACC AGGCAGCTAC TTATCTGCCA CGAAGATATG CGTGGCAGAA  
 TGGACAACAT GACTAAGTTA ATTAGTGACG TATTAGAAC ATTGGCTGGA GGTAGTAGCA AAGCACCCAC  
 CGAGAAAGTCG GATATTGATA CGATGCCCTCC TTCTAATGAT GCTGGTTCTG AGCCACAGCC CCAACCTAGC  
 GAAAGTAAAC CACCCGAGCA ACCCTCTCCC GAACCCGAAA AAGACTCTTC TAGTAAACCA TCAGATCAAC  
 CTACTCCCGA ACCCGAAAAA GGCTCTTCTA GCAAACCCCG TACAGATATC TTTAGTGGTT TACGTAATAA  
 AGAAATTAAT TTTTGAAAGA ACTGTTGGAG CATTTATCCA ATATTATTAT TTAATTTAAA TCAATTGAGT  
 TAATGTAATA ACTTTTACA TATATTTTGC TCTAGTCCGA AATAGGAAAT TAGCAAAAAA TAATGATTAT  
 TATATATTAA TGTTTTAACT TAATAATTAA TTTATAAAAT ATTTATTGTC ACATCCGTTT TTTATCACGT  
 TATCGTACGT GGAGGTAGT TATGGATGTT TTTATCATTA CTTTTTGTA TAGTAGTAAA TAGTATCACA  
 GCAGATTAA TTACTTCTGT GATATACAAT ACATGTATTA AGGATTACCC GCACCCAAAA ATAATATCGT  
 GATCTGTAAT ATATAAAAAAT AAATACCATA CTATATGTTT ATATCACCAA TCATGGTAAA AATAGTGTTT  
 GTGATGAATTC  
 EcoRI

Fig. 10B

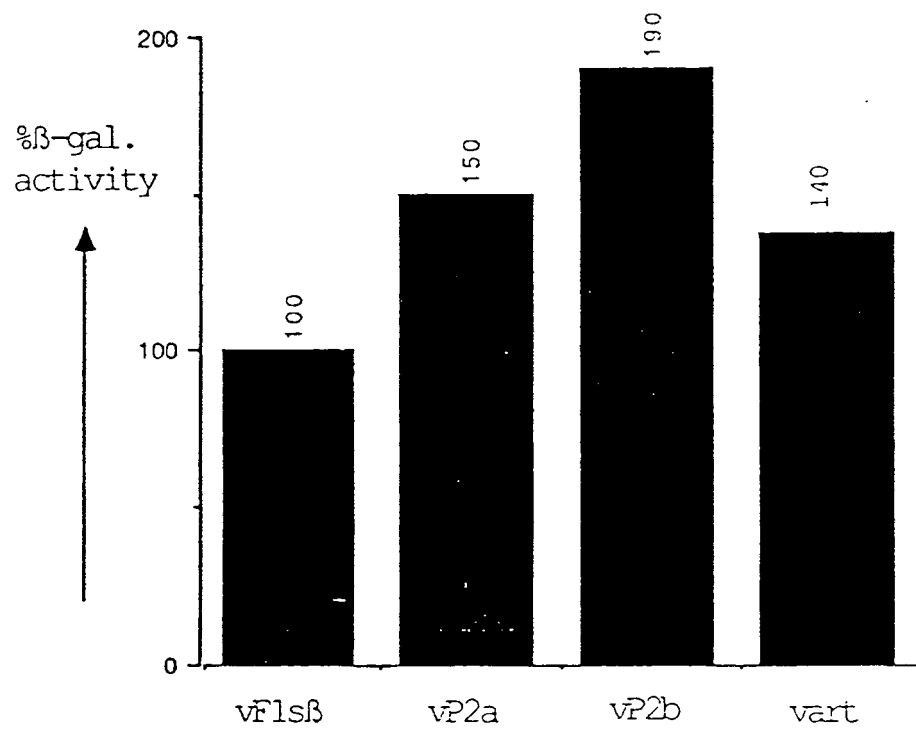


Fig.11

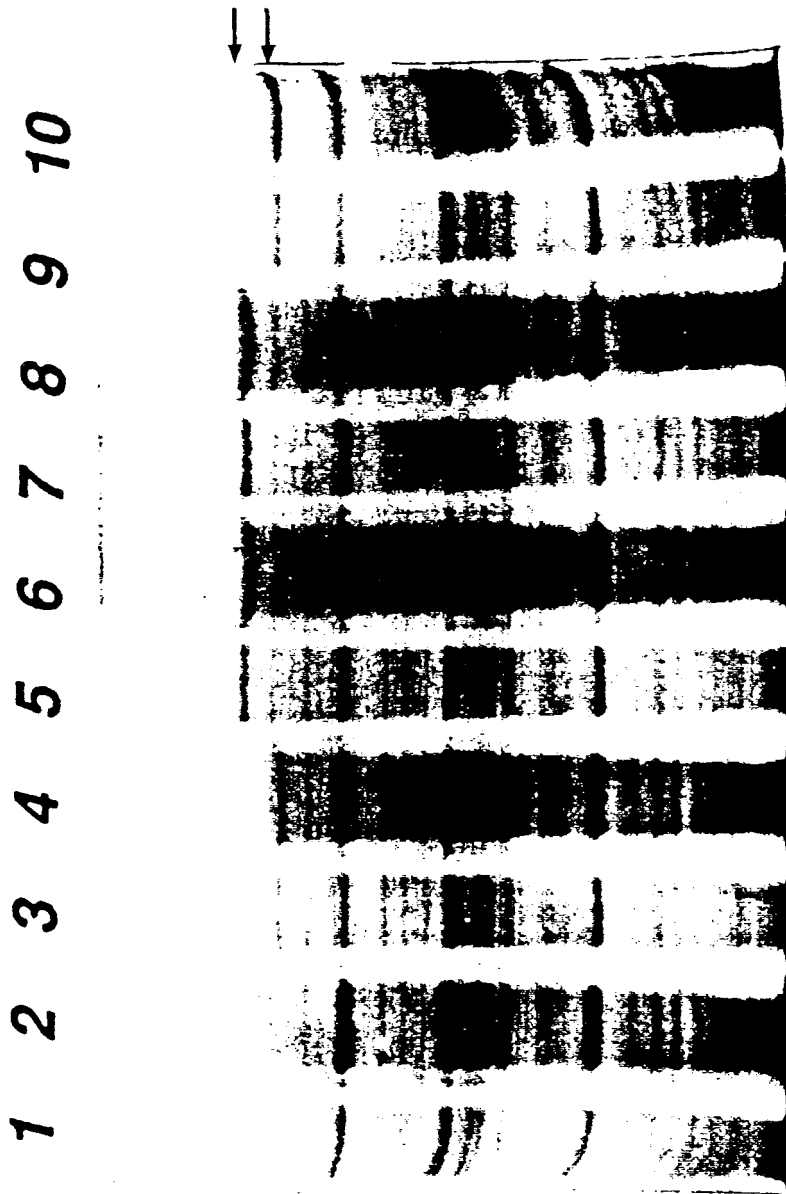


Fig.12

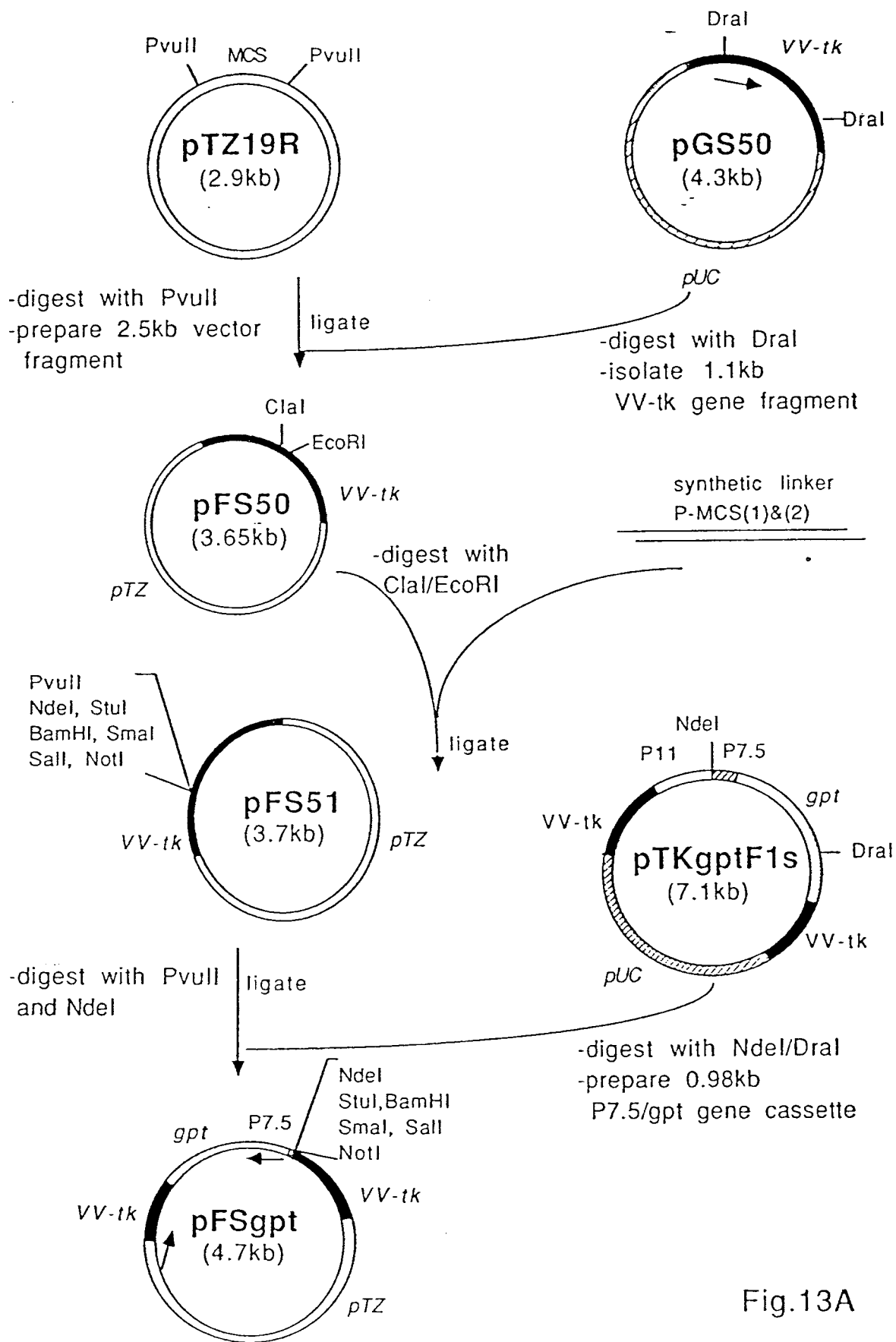


Fig.13A

Fig. 13B

Sequence of the multiple cloning site of pFSgpt

CATATG AGGCCT GGATCC CGGGTCGAC GCGGCCGC TAACTGACTGATTTTTCICAATT  
*Nde I*      *StuI*      *BamHI*      *SmaI*      *Sall*      *NotI*

Fig.14A

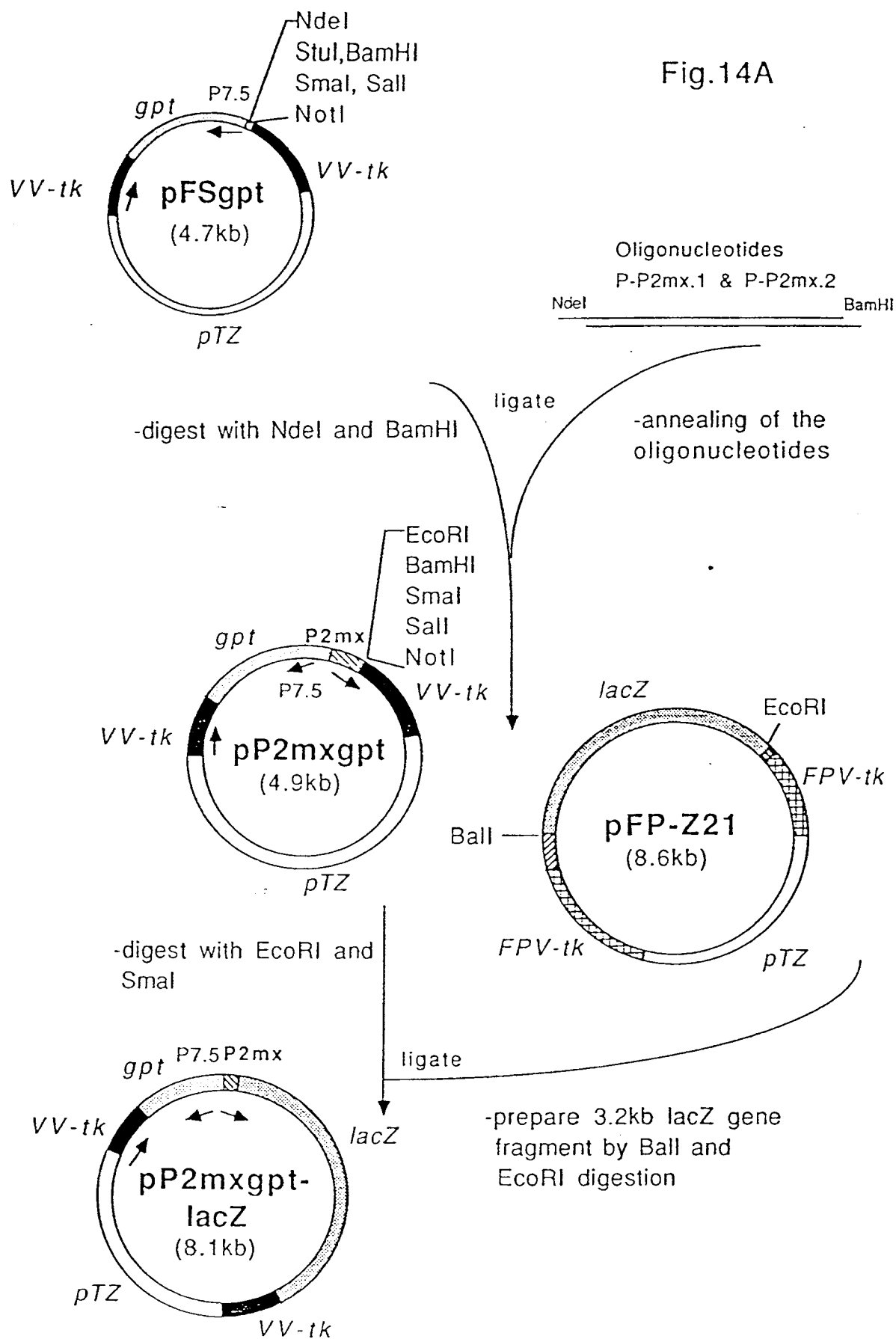




Fig. 14B

Sequence of the multiple cloning sites of the insertion plasmids, pP2mxgpt

ATGAATTC GGATCC CCGGTCGAC GCGGCCGC TAACTGACTGATTTTTCICAATT  
*EcoRI* *BamHI* *SmaI* *Sall* *NotI*

5'-AATGAGTAGAAAATACTATTTTTATAGCCTATAAAATC ATG GAA AAG...//...TCC TTA CAT ATG GTT CGT-3'  
P2-gene/polylinker sequences  
lacZ

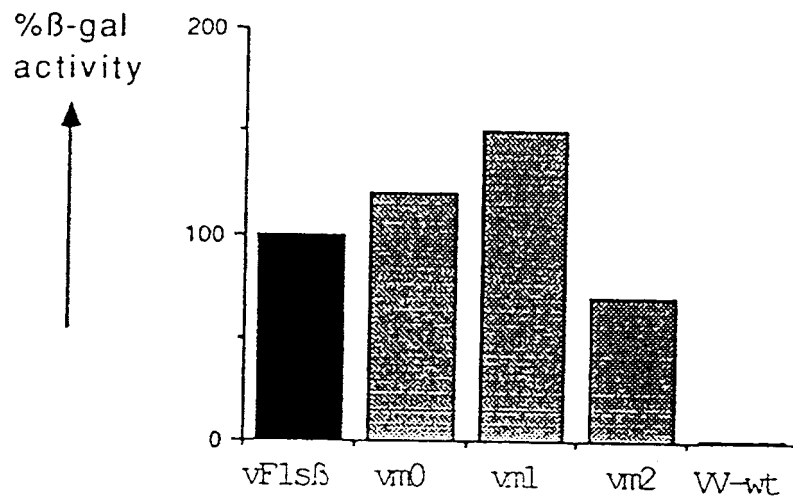
5'-AATGAGTAGAAATACTATTTTTATAGCCTATAA ATG AAT TCC GTT CGT-3'  
 EcoRI  
 lacZ

5'-AATGAGTAGAAAATACTATTTTTATAGCCTATAAATC ATG AAT TCC GTT CGT-3'

5'-AATGAGTAGAAAATACTATTIIGTTTTATAGCCTATAAATC ATG AAT TCC GTT CGT-3'  
 insertion  
 EcoRI  
 lacZ

50

a) late promoter activity



b) early promoter activity

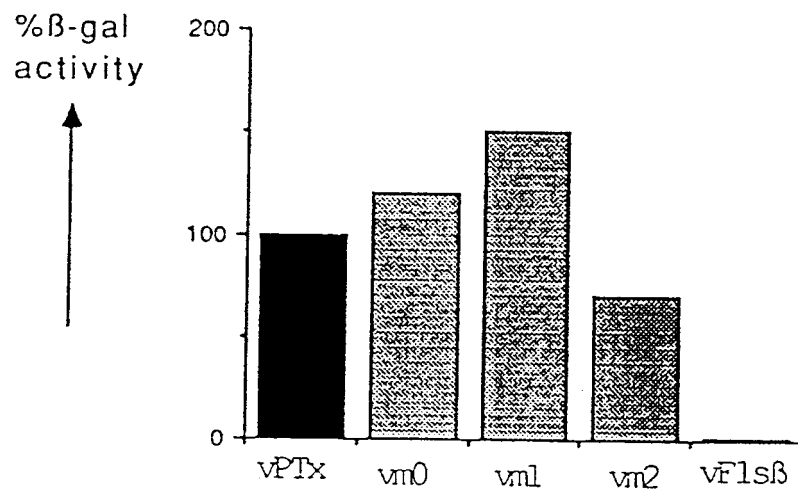


Fig.16

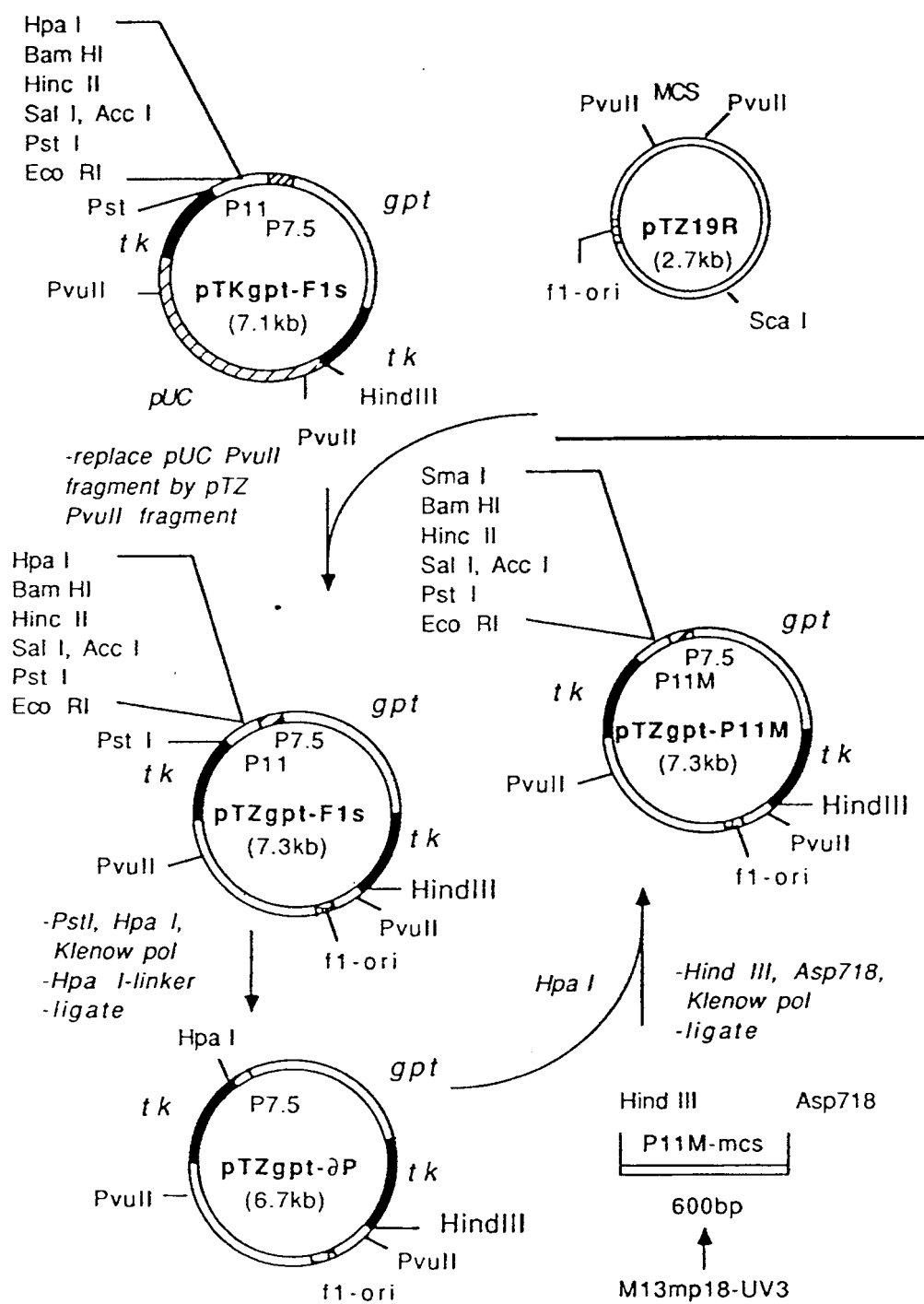


Fig 17A

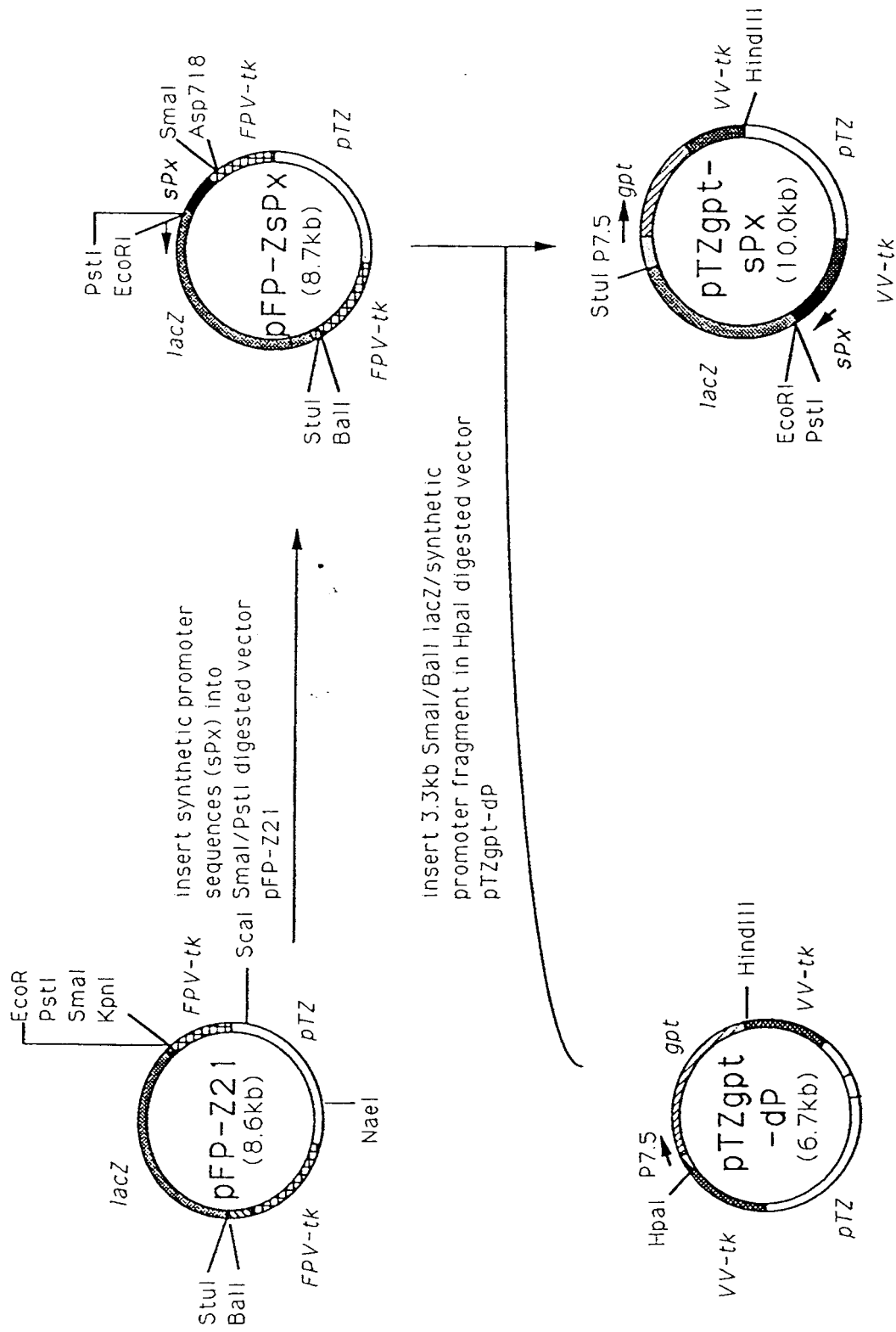


Fig. 17 B

*P11wt*

5'-GAATATATGT ATGTA<sup>EcoRI</sup>AAAAT ATAGTAGAAT TTCATT<sup>BamHI lacZ</sup>TGT TTTT<sup>lacZ</sup>TTCTAT GCTATAAAATGAATTCCTGCAGGTCGACTCTAGAGGATCCCGTC-3'

*P11m*

5'-ATTTAGAAT ATATGTA<sup>EcoRI</sup>TGT AAAAAATATAG TAGAATTCA TTTTGTTTTT TTCTATGCTATAAAATAAGAATTCCTGCAGGT...CATATG-3'<sup>58bp lacZ</sup>

*sP11*

5'-GGGTTAGAAT ATATGTA<sup>PstI EcoRI</sup>TGT AAAAAATATAG TAGAATTCA TTTTGTTTTT TTCTATGCTA TAAATAGGCT GCAGGAATTCCTTACATATG-3'<sup>lacZ</sup>

*s4b*

5'-GGGGTTACGT CTCTTTAGGT ACTTATT<sup>PstI EcoRI</sup>TG ATACGTTACA AGTAAAAAAC TATCAAATAT AAATAGGCTG CAGGAATTCCTTACATATG-3'<sup>lacZ</sup>

*sart*

5'-GGGAAGCTTT TTTTTTTTTT TTTTTTTGGC ATATAAATAG GCTGCAGGAATTCCTTACATATG-3'<sup>PstI EcoRI lacZ</sup>

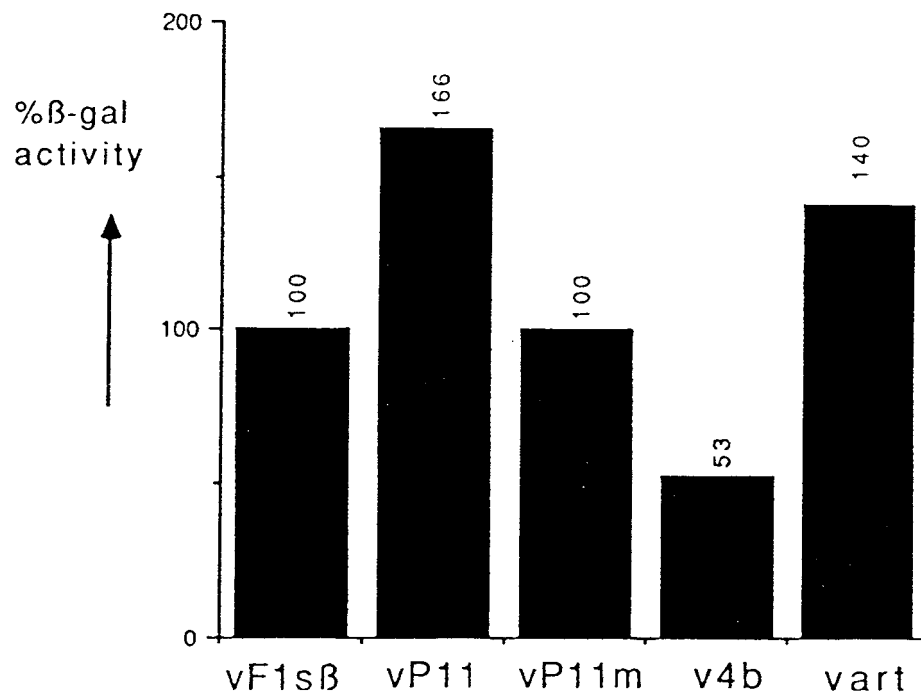


Fig. 19



DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
D,X	EP-A-0 314 569 (TRANSGENE S.A.) * the whole document * ---	1,7,29	C 12 N 15/86 C 12 N 15/85 C 12 N 15/39 A 61 K 39/00
D,A	WO-A-9 002 191 (APPLIED BIOTECHNOLOGY) * see specially p 36 line 4 to 28; the whole document * ---	1,2,7,14,29	
A	WO-A-8 912 684 (NATIONAL RESEARCH DEVELOPMENT CORPORATION) * the whole document * ---	1,7,29	
D,A	WO-A-8 903 429 (HEALTH RESEARCH) * the whole document * ---	1,7,29	
A	WO-A-9 012 882 (HEALTH RESEARCH, INC.) ---	1,7,29	
D,A	EP-A-0 284 416 (NIPPON ZEON CO., LTD) * the whole document * -----	1,7,29	
			TECHNICAL FIELDS SEARCHED (Int. Cl.5)
			C 12 N
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 15-04-1992	Examiner CHAMBONNET F J
<b>CATEGORY OF CITED DOCUMENTS</b> X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			